THE EFFECT OF PITUITARY EXTRACTS ON MELANOPHORES IN ISOLATED ELASMOBRANCH SKIN

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SUMMARY.

Alkali-treated B-containing extracts from ox pituitary exhibit increased strength (potentiation) and increased duration of response (protection) both *in vivo* and *in vitro*.

Protection is in part explicable by the decreased metabolic destruction of the treated hormone.

INTRODUCTION.

Many cold-blooded vertebrates change colour in response to external stimuli. Anurans and elasmobranchs darken when placed in black tanks with overhead illumination, as a result of dispersion of melanin granules in dermal and epidermal melanophores; in white tanks with overhead illumination, they pale owing to aggregation of melanin within the melanophores. The eye is the receptor for this response and the pituitary is the co-ordinator (reviewed by Hogben, 1924; Waring, 1942).

Hypophysectomised animals or intact ones, pale owing to sojourn on a white background, exhibit temporary darkening when injected with pituitary extract. The responsible hormones, variously called B, intermedin, or M.S.H., are manufactured in the intermediate lobe (Hogben, 1924). The chemistry of these hormones from pig, ox and man has recently been elucidated (Porath *et al.*, 1955; Harris and Roos, 1956; Benfey and Purvis, 1955; Geschwind *et al.*, 1956, 1957; Harris and Lerner, 1957; and Harris, 1959). From the pig two have been isolated, α and β, from the ox one, β, and from man one, β. Pig α has 13 amino acid residues and pig β has 18; ox β is similar to that from pig except that one glutamic acid of the pig β is replaced by serine; human β has 22 amino acid residues.

When simple, unfractionated, B-containing extracts from ox, frog and dogfish, but not from pig, are treated with alkali, their melanophore excitant properties are modified in two ways (Landgrebe *et al.*, 1941, 1943 and 1955); (a)

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the strength, or potency, of the extract is increased – this change in the extract we have called its *potentiation*; and (b) there is an increased duration of response to sub-maximal doses – this change in the extract we have called its *protection*, because there is evidence that it is due to the hormone's increased resistance to destruction in the body. Pig material exhibits protection, but no potentiation (Landgrebe and Mitchell, 1954); human material has not been tried. There seems to be no record of whether completely pure ox hormone is potentiated by alkali, but *some* partially purified extracts of ox are not (Landgrebe *et al.*, 1943, 1954 and 1955). So it seems likely that we still have to think in terms of inactive associated precursors (Waring and Landgrebe, 1941) in seeking an explanation of potentiation; this despite the temptation to speculate on the basis of the known effects of alkali on the closely related corticotrophin (Shepherd *et al.*, 1956; Harris and Lerner, 1957; Astwood, pers. comm.). In short, the chemistry of potentiation (and of protection) is at present unknown.

The present paper describes experiments designed to show whether protection and potentiation are exhibited by melanophores separated from central connection. Initial experiments were performed on skin cut from *Rana* and *Xenopus*, but the later experiments reported here were performed on *Scyllium* (dogfish) skin because in my hands all phases exhibited in the intact animal were more faithfully followed *in vitro* with this material. With the dogfish it is easy to remove only a thin layer of skin by stripping from the fin rays and probably the easy penetration of fluids played a big part in the success of this preparation.

Materials and Methods.

The melanophore indices (m.i.) are according to Hogben and Slome (1931). Initial and caustic-treated extracts were prepared as previously (Landgrebe and Waring, 1950). The initial extract contained 2 I.U. per ml.; caustic-treated extracts were prepared from the initial extract without change of volume.

Dogfish were selected for low melanophore density to make reading of m.i. easier, and, after preliminary experiments, to show that the melanophores behaved normally, the fish were hypophysectomised (Waring, 1938). Skin was cut from the pectoral fin and immediately immersed in Young's (1933) elasmobranch saline (sea water 610 ml., distilled water 390 ml., urea 29 gm., pH 7·5). The epidermis and dermis together were quickly stripped from the finrays and cut into pieces about 2 × 2 mm. Each piece was placed in a separate watch glass with 0·5 ml. of saline, or of saline plus pituitary extract. Control pieces of skin kept in saline throughout the experiments revealed that saline would (a) maintain the melanin in an aggregated state (m.i. = approx. 1) during the period of the experiment, and (b) retain the reactive properties of the melanophores unimpaired over the period of the experiment. The latter was tested by periodically adding pieces of skin from the control pool in saline to pituitary at concentrations known to raise the m.i. to a definite value; in addition, skin whose pigment had been dispersed by pituitary was immersed in fresh saline until the melanin was fully aggregated, and was then placed in pituitary solutions of known potency. All experiments showed that no measurable diminution in performance of melanophores took place in 24 hours. The more obvious errors likely to occur in this type of experiment are due to differences in sensitivity between melanophores from different fish

and between different areas of the same fish. The first was controlled by complete sets of experiments extending over three or four weeks from the same fish; the latter by having at least four pieces of skin at each pituitary concentration in any one experiment. Furthermore, it was an easy matter to read the same melanophores at the same loci on each occasion during the course of one experiment. A dissecting dish covered the watch glasses between readings, both to minimize any interference of strong light and to retard evaporation. All experiments were at room temperature.

EXPERIMENTAL OBSERVATIONS.

Whole animals.

Our first observations (Waring and Landgrebe, 1941; Landgrebe and Waring, 1941) on the two effects of alkali-treatment on extracts employed intact and hypophysectomised *Xenopus* as the test material. When it became clear that *Scyllium* was more suitable material for these isolated skin experiments, it was necessary to test whether the whole fish, intact or hypophysectomised, exhibited the two effects under review. *Scyllium*, intact or hypophysectomised, exhibited the potentiated and protected nature of alkali-treated extracts when compared with untreated extracts. The chief points of interest that emerged were: (a) for a given treated extract, compared with its untreated counterpart, *Scyllium* recorded greater potentiation and less protection than *Xenopus*; (b) immediately after hypophysectomy *Xenopus* is less sensitive than intact animals, and over a period of weeks its sensitivity increases and transcends that of the intact animals (Landgrebe and Waring, 1941, 1944). With *Scyllium* there is an immediate increase of sensitivity after hypophysectomy, a finding consistent with earlier observations on this fish (Waring, 1938).

Isolated skin.

Melanophores in skin isolated from *Scyllium* and placed in untreated extract exhibit melanin dispersion; this reaches a maximum in about two hours when aggregation of the melanin starts and proceeds to completion. Within a limited range, discrimination between doses is possible (Fig. 1). The sequence of melanophore changes disclosed by Fig. 1 implies that under these experimental conditions the strength of hormone in the watch glasses declines during the experiment and this is confirmed by placing fresh pieces of skin, with aggregated melanin, in the solution. So in rough fashion, the saline with its added pituitary mimics the circulation of the whole animal after a single injection.

If in these experiments treated extract is used, the sequence of events is similar except that (a) less extract is needed to attain the peak m.i., and (b) the action is very prolonged (Fig. 1). This shows clearly that melanophores severed from the central organisation of the body can detect the potentiated nature of the treated extract. They also show the protected nature of the extract. While we cannot be certain that the increased duration of response to treated extract shown by the studies *in vitro* and *in vivo* is due to precisely the same

cause, the *in vitro* preparation does permit direct test of certain possible explanations of protection:

i. *Does exposure to treated hormone modify the melanophore itself so that when the excitant is removed the melanophore cannot aggregate its pigment so rapidly?* To test this, skin was immersed in a series of doses of treated and simple extract, matched by trial to give the same peak m.i. After the peak was reached the skin was washed in saline and left in fresh saline. There was no measurable difference in the time needed for aggregation of melanin in the skin from the two extracts. It appears therefore that treated extract leaves no continuing effect on the melanophore after its removal.

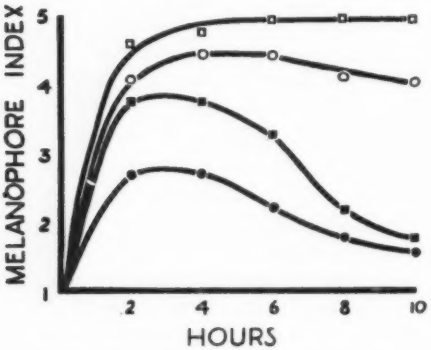

Fig. 1. Strength of untreated extract is expressed in international units (Landgrebe and Waring, 1950) per ml. of bathing fluid.

Treated extract, prepared from the same initial untreated extract, contained precisely the same amount of original pituitary substance.

● — ● Plottings from skin in saline containing 0·0006 i.u. untreated extract per ml.

○ — ○ Plottings from skin in saline containing treated extract which before treatment assayed 0·0006 i.u. per ml.

■ — ■ Plottings from skin in saline containing 0·001 i.u. untreated extract per ml.

□ — □ Plottings from skin in saline containing treated extract which before treatment assayed 0·001 i.u. per ml.

○ was still at m.i. 4·0 24 hours later and melanophores were still active.

□ was still at m.i. 5·0 24 hours later and melanophores were still active.

(The reactivity of both skin samples, ○ and □, was verified by placing part of each in saline only, when the m.i. promptly fell to 1·0; and the remaining parts left in the original solution until they came down to m.i. =1·0. The latter were then placed in fresh pituitary extract to which they reacted.)

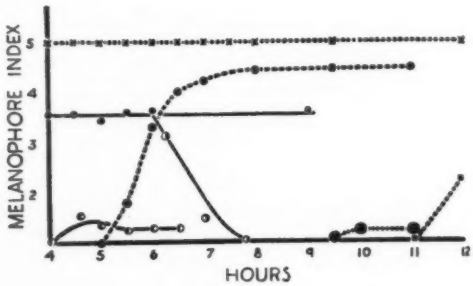

Fig. 2. Same *treated* pituitary extract as for Fig. 1. *All* skin samples detached from fish at same time and kept in saline until used.

A. ● — ● Plottings from skin placed in saline containing extract which, before treatment, assayed 0·0001 i.u. per ml.

B. ◑ — ◑ Plottings from pieces of skin cut at 6 hrs. from samples A and placed in saline alone.

C. ◐ — ◐ Plottings from skin placed in same watch glass as A, 4 hr. after start of experiment.

D. x — x Plottings from skin placed in saline containing extract which, before treatment, assayed 0·0005 i.u. per ml.

E. ⊗ — ⊗ Plottings from skin placed in same watch glass as sample D, 5 hr. after start of experiment.

F. ◎ — ◎ Plottings from skin placed in same watch glass as D, 9¾ hr. after start of experiment.

G. ■ — ■ Plottings from skin (in saline alone since beginning of experiment) placed in *new* pituitary solution of strength D, 11 hr. after start of experiment. This shows skin has retained its reactivity.

Both x and ⊗ had returned to m.i. =1·0, 9 hr. after the last plotting shown here.

ii. *Does treated hormone owe its protected properties in part to being less easily destroyed by tissues?* This was tested in two kinds of experiments:

(a) Using the same fish material, and dilution of the same extract which previous experiment had shown to give a peak m.i. of 3·5 (i.e., a submaximal response), a series of watch glasses was set out containing extract but no skin. At intervals skin was added to an aliquot to test the strength of the extract. Neither treated nor untreated extract deteriorated measurably during the time taken for an average experiment (12-15 hours). An exactly comparable experiment was set up but with skin in the watch glasses from the start; at intervals a second piece of skin was added to test the strength of the extract. There was a measurable loss of activity in both kinds of extract containing skin, but the treated extract maintained its power to evoke expansion longer than the untreated.

(b) Unfortunately, discrimination between doses by isolated skin in the experiments (a) above is only in a limited range and not better than 25 p.c., so the above experiment could at best only furnish a roughly quantitative answer. To get a more precise answer the following experiment was performed. Undiluted treated and untreated extracts were left standing in watch glasses overnight, with and without pieces of skin; the contents of the watch glasses were then assayed on *Xenopus* (Landgrebe and Waring, 1944, 1950). There was very little loss of activity in either treated or untreated samples without skin; thus both extracts, undiluted, are stable when exposed to air under the conditions of the experiment. With skin immersed in the extract at least 75 p.c. of the activity of untreated extract, and about 50 p.c. of the treated, were destroyed. The skin can therefore bring about considerable destruction of a strong extract in 18 hours, and treated extracts are more resistant than simple ones. It is unfortunate that the relative insensitivity of the *Xenopus* test makes it impossible to get unexceptional data with the same dilutions as used in the isolated skin experiments.

iii. *Does treated hormone owe its protected properties in part to an ability to maintain itself in higher concentration at the melanophore surface than in the general bathing fluid?* Three kinds of experiment were performed:

(a) Skin was placed in saline with treated extract, and, when the melanin had dispersed maximally, the extract was carefully replaced by saline alone; the skin was not washed in saline as in (i) above. The pigment immediately began to aggregate in response to the reduced hormone in the bathing fluid.

(b) Skin with aggregated melanin was placed in treated extract of strength just insufficient to start an immediate response; the melanophores did not accumulate activity sufficient to evoke dispersion of melanin when left for long periods.

(c) Skin was placed in saline containing treated extract and time was allowed for the m.i. to reach a maximum; this maximum was maintained for long periods. Then fresh pieces of skin were placed in the same watch glass at various intervals. The results from a typical experiment are graphed in Fig. 2. The graph shows that, when conditions in the watch glass were maintaining an m.i. of 3·5 in the original piece of skin, there was only sufficient free excitant to raise the m.i. in the fresh piece of skin to 1·5. By subdividing the skin under observation it was confirmed that a maintained index would rapidly return to base if saline were substituted for pituitary extract, and would return to base eventually if the original extract were left bathing the skin.

The results of experiment (c) are open to two interpretations. Either less treated hormone is needed to maintain the m.i. at 3·5 than to raise it from 1 to 3·5, or the treated hormone remains in higher concentration at the melanophore surface than in the bathing fluid when the hormone concentration in the bathing fluid begins to fall. Choice between these possibilities, by direct test of the first using untreated extract, proved impracticable because the fall in hormone level was too rapid under these conditions after peak m.i. had been attained. The only observations favouring the second possibility, in preference to the first, are Wright's (1955) using untreated extracts. Wright had his skin in extracts in a photometer, and under these conditions, possibly owing to restricted contact with air, the extracts did not deteriorate during the course of an experiment. He found that the same concentration is needed to raise the m.i. to a given figure, as to maintain it there; it is conceivable, however, that treated and untreated extracts behave differently in this respect.

DISCUSSION.

Alkali-treated ox pituitary extract exhibits potentiation and protection when tested on skin melanophores *in vitro*. So both these properties of treated extract can be exhibited peripherally without central mediation.

Further understanding of potentiation will depend chiefly on analyses for amino acid sequences of various extracts, notably pig and ox, after alkali treatment.

Three possible explanations of the protected nature of the treated extract, as shown *in vitro*, have been explored. There is no reason to suppose that melanophores are modified by contact with treated extract so that subsequently their pigment takes longer to aggregate. The evidence for an increased concentration of hormone at the melanophore surface compared with the concentration in the bathing fluid is equivocal. There is good evidence that treated hormone is less readily destroyed than untreated hormone when in contact with skin, and taking into account Wright's (1955) observations, together with the present ones, there is a clear suggestion that the destruction of hormone by skin is a reaction requiring oxygen.

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TISSUE INJURY BY HIGH FREQUENCY ELECTRIC CURRENT: OBSERVATIONS WITH THE SANDISON-CLARK EAR-CHAMBER

I. INJURY TO SMALL BLOOD VESSELS

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SUMMARY.

High frequency current has been employed to produce minute injuries to selected blood vessels within Sandison-Clark rabbit ear-chambers. There are two principal findings.

First, at the surface of injured endothelial cells transparent blister-like structures appear which, after increasing in size, separate from the cell as transparent globular bodies, floating freely in the plasma. It is concluded that these structures are produced by the damaged endothelial cells.

Second, the area of vascular damage apparent within one hour of injury is increased fivefold by 24 hours. It is suggested that the initially injured cells may liberate diffusible chemical factors which secondarily injure the surrounding endothelial cells. Alternatively, or perhaps additionally, this phenomenon may be due to initial latent cell injury which only gradually becomes visible with the passage of time.

INTRODUCTION.

In the study of inflammation, direct microscopic study of experimentally injured living tissue has proved itself a worthwhile method for over a century (Addison, 1843; Waller, 1846; Cohnheim, 1889). There is an absence of cytological distortion caused by tissue processing procedures associated with section cutting, and the dynamic aspects of cellular and tissue changes are clearly revealed. The possibilities of the method were greatly extended when Sandison (1924, 1928) introduced the rabbit ear-chamber. Then a thin section of living mammalian connective tissue, protected from dehydration and bacterial infection, became available for high resolution light microscopy. Further, the sequelae of a variety of types of tissue injury could be observed over extended periods of time.

Many methods of damaging tissue with rabbit ear-chambers have been developed and each has contributed knowledge of tissue responses to injury. The technique developed for the present work (Buckley and Semkiw, 1959) introduces new possibilities, enabling one to inflict tiny aseptic injuries at any site

within the ear-chamber. Thus the experimenter is able to injure selectively small groups of the various types of cells without obscuring details of tissue changes as occurs with grosser injuries.

The present paper deals with the series of changes observed following injury by high frequency current to small blood vessels within the rabbit ear-chamber.

Materials and Methods.

Perspex ear-chambers of a modified round table type with 40μ thick mica covers were installed in rabbits' ears. A 35 mm. camera and an electronic flash tube were employed for photomicrography. Precise details of improved techniques for ear-chamber manufacture, installation and photomicrography are described in a separate communication (Buckley, 1960).

Small injuries to the tissue within the ear-chamber were made by means of a radio-frequency generator. This apparatus and the technique employed have been fully described (Buckley and Semkiw, 1959). In brief, a fine stainless steel electrode, connected to the radio-frequency generator, is positioned to lie on the mica cover of the ear-chamber over the "target" vessel. Before a discharge button is pressed, controls on the generator chassis are set to give an R.F. wave-train of the required intensity and duration. The technique is capable of producing very small injuries at any chosen site within the ear-chamber.

RESULTS.

Damage to vascular connective tissue within ear-chamber.

Early changes. Discharge of the high frequency generator caused immediate contraction of vessels within a circular area of approximately 120μ diameter. During the following 5-10 seconds considerable recovery in shape of the affected vessels occurred, but a degree of contraction with wrinkling of the vessel walls remained (Fig. 1). As the vessels contracted at the instant of injury, all surrounding structures moved away from the centre of damage, then rapidly returned to their original positions. Sometimes, however, cells very close to the centre of injury were permanently displaced. At the time of injury red cells in the target vessel were converted into a larger number of small fragments of variable size and shape (Fig. 2).

Details of vessel walls, normally quite clear (Figs. 3 and 4), soon appeared blurred, and there was often obvious swelling of the endothelial cells (Figs. 5, 6 and 7). The grossly swollen endothelial cells developed a mottled appearance due to numerous small cytoplasmic vacuoles with adjacent small dark granules (Figs. 7 and 8).

Within 5-10 minutes of injury the red cells and red cell fragments, in and immediately adjacent to the damaged capillary segment, became concentrated within the vessel lumen (Fig. 5).

At about the same time, colourless, transparent, amorphous material in round or oval form began to appear within the lumen of the injured vessel (Figs. 5 and 6). When occurring in close relation to the side wall of a vessel (as

distinct from the upper and lower walls which cannot be seen in optical section), the transparent material appeared as a sharply defined blister-like structure with its base at the damaged endothelial surface, the remainder projecting into the vessel lumen (Fig. 6). Observed in this way it could be seen that a gradual but steady increase in size took place, the blisters sometimes becoming quite large. Finally, after developing an hour-glass constriction near their base, blisters separated from their site of attachment and floated away as round or oval transparent globules. The final picture obtained, however, greatly depended on the state of blood flow. In most of these experiments blood flow was not accelerated following the small injuries. The two extremes in states of blood flow are described in detail below; however, many gradations between these extremes were observed.

(i) Rapid flow.—When blood flowed rapidly through the damaged vessel, blisters were at first deformed in the direction of flow, then separated from the underlying endothelium while still small; thus no free floating globules accumulated, as they were soon carried off in the blood stream. Masses of platelets, leucocytes and red cells rapidly accumulated on the injured endothelium to form a thrombus. This aggregation of material frequently masked the few blisters and free globules present, and made identification difficult (Fig. 9). Leucocytes which adhered to the injured endothelium lost their rounded form, and moved in an amoeboid fashion along the endothelial surface. Subsequently most leucocytes migrated through the vessel wall into the surrounding tissue where they continued their active amoeboid movement (Fig. 10).

(ii) Slow flow.—Under conditions of slow or absent blood flow the damaged segment gradually filled with transparent blisters and globular bodies. In these circumstances, platelets, leucocytes and red cells infrequently made contact with damaged endothelium and therefore did not accumulate. Leucocytes and erythrocytes passing slowly through such a globule-filled vessel were sometimes seen to be deformed as they moved between adjacent globules. Whenever the formed elements of the blood collided with the transparent globular material they failed to stick to it, and globular bodies did not adhere to one another.

Red cells remaining in the region of injury, together with those subsequently accumulating, coalesced and merged with one another to form a homogeneous mass of opaque red material within the vessel. Globules were sometimes seen projecting into this opaque red material (Fig. 8).

Careful observation showed transparent material outside the vessel walls; however, under these circumstances the material was not in the form of blisters or globules, but conformed to the shape of the cleft-like perivascular space between vessel wall and surrounding connective tissue fibres (cf. Figs. 4 and 7).

Late changes. The area of damaged tissue seen one hour after injury was greatly extended by 24 hours. For example, in one experiment the area of damaged tissue at 15 hours was approximately five times that seen 40 minutes

EXPLANATION OF PLATES

Fig. 1. Photomicrograph of capillary within ear-chamber showing residual wrinkling of endothelium following injury by high frequency electric current. x 600.

Fig. 2. Photomicrograph illustrating the intravascular fragmentation of red cells which occurred at the time of initial injury. Fragments varied in size and were generally globular or ovoid while leucocytes and platelets appeared to be unchanged. x 600.

Fig. 3. Photomicrograph of "target" area and surrounding connective tissue within ear-chamber prior to injury. The blood within the principal vessel was moving from left to right. Various connective tissue cells surrounded the blood vessels. x 375.

Fig. 4. Higher power view of target area before injury. The electrode was placed at X. Note the well-defined outlines of undamaged endothelial cells lining the blood vessels, the erythrocytes and leucocytes within the blood vessels and the coarsely granulated macrophages outside the vessels. x 700.

Fig. 5. Photomicrograph of same area as in Fig. 3, ten minutes after injury, showing endothelial cell swelling, early blister formation and concentration of red cells in the adjacent segment of vessel. Similar changes occurred in the vessel on the left, but no changes were visible in the vessel on the right. x 375.

Fig. 6. Photomicrograph of a capillary 12 minutes following injury, illustrating gross endothelial cell swelling and blister formation at the injured cell surface. The transparent globular bodies to the right caused deformation of passing red cells. x 700.

Fig. 7. Photomicrograph of area shown in Fig. 4, illustrating swelling and mottling of endothelial cells together with blister and globule formation 20 minutes after injury. Adjacent red cells were seen in rouleaux formation while the flow in the larger vessel continued unaffected. x 700.

Fig. 1

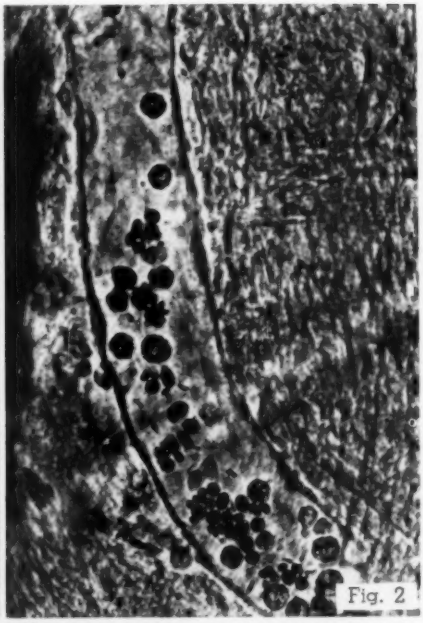


Fig. 2

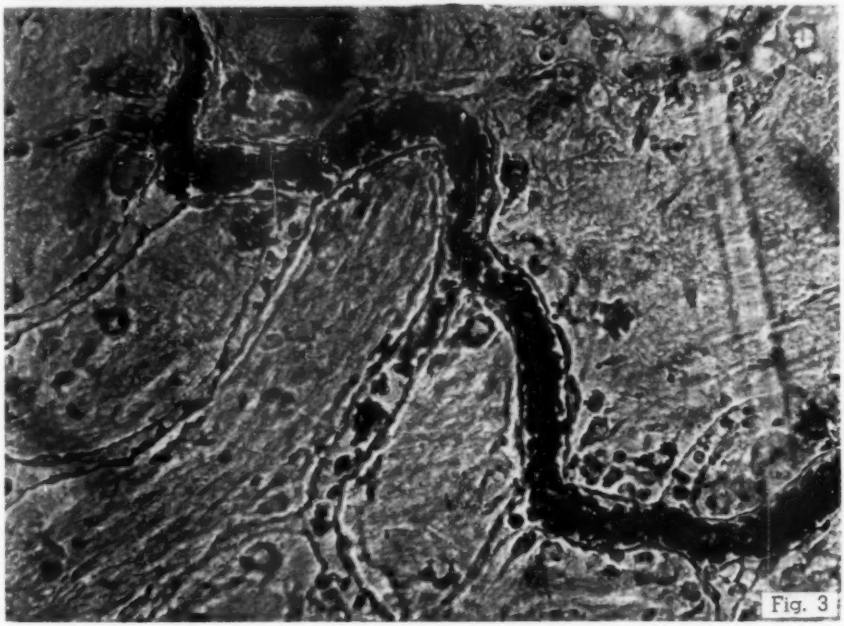


Fig. 3

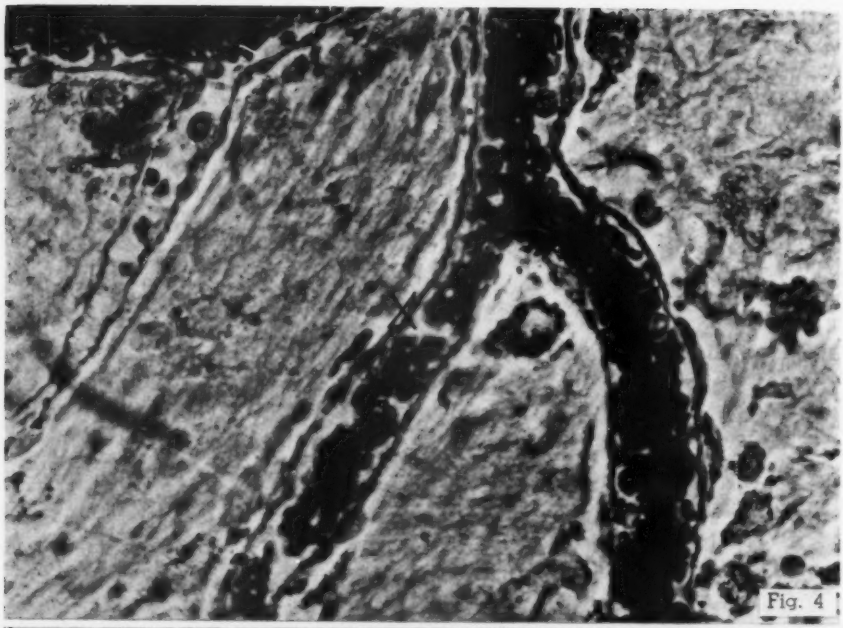


Fig. 4

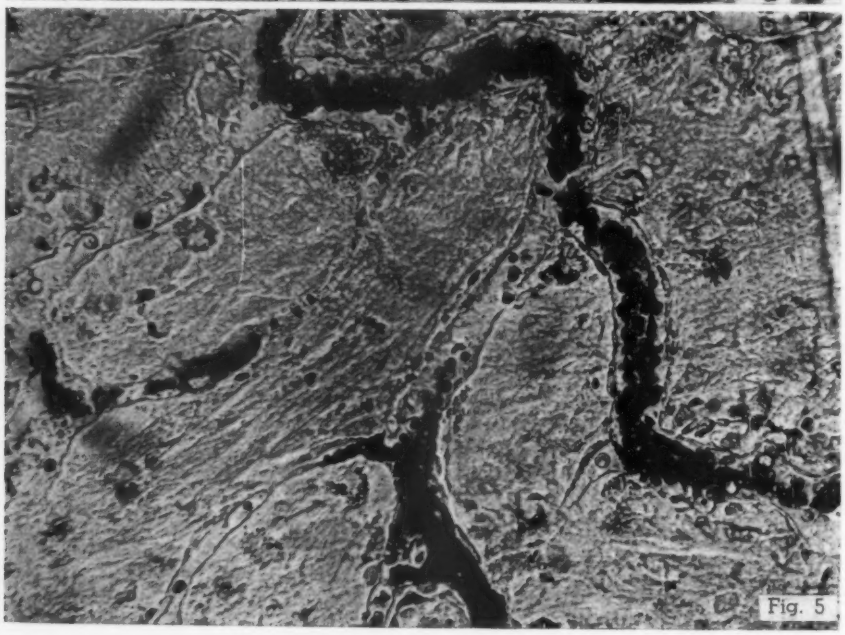


Fig. 5

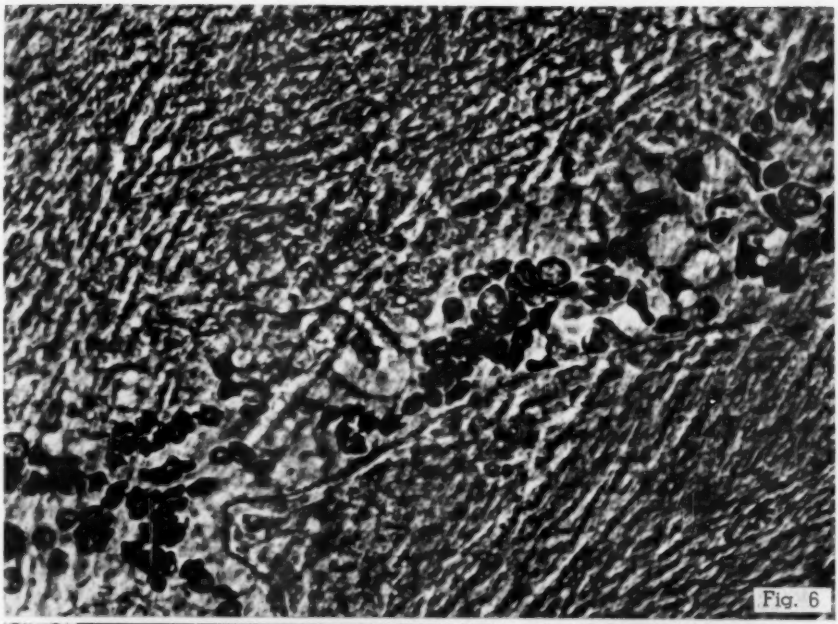


Fig. 6

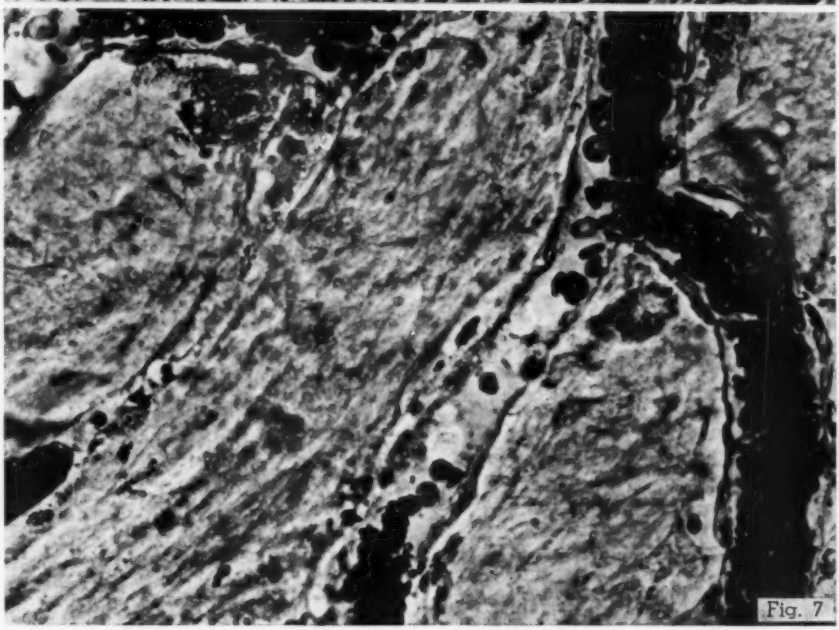


Fig. 7

EXPLANATION OF PLATES

Fig. 8. Thirty-five minutes following injury the transparent globular material projected into the surrounding laked red cells and could thus be seen more clearly. A small amount of transparent material lay outside the vessel wall adjacent to the swollen mottled endothelial cell (see arrow). Note similar changes within the vessel on the left and the absence of any sign of damage to vessel on the right. x 700.

Fig. 9. Photomicrograph of a venule illustrating the consequences of rapid blood flow following injury. All but a few blisters and globules (indicated by arrows) had been swept away in the blood stream, while platelets, erythrocytes and leucocytes had accumulated on the injured endothelial surface to form a thrombus. x 700.

Fig. 10. Photomicrograph of vessel shown in Fig. 6 illustrating further endothelial cell swelling and the accumulation of a considerable number of actively motile leucocytes 65 minutes after injury. Some of these were moving within the vessel lumen while many others had migrated through the vessel wall into the surrounding connective tissue. x 700.

Fig. 11. Injured vessels 16 hours after injury (cf. Figs. 5, 8) demonstrating the increased degree and extent of vascular damage which eventually occurred. Note collections of laked red cell and transparent globular material outside damaged vessels. x 375.

Figs. 12 *and* **13.** Higher magnification of portions of field shown in Fig. 11 to illustrate details of the various changes. In some places the blood vessels were contracted and devoid of contents, while in others they contained laked red cells and small amounts of globular material. Outside the vessels there were laked and intact red cells, transparent globules and an increased number of macrophages. Fig. 13 shows an adjoining undamaged vessel containing a number of leucocytes, two of which each contained a single small vacuole. x 700.

Fig. 14. Photomicrograph of vascular connective tissue within the ear-chamber of a rabbit which had been dead for 24 hours. The tissue showed transparent globular bodies similar to those observed in experimentally injured vessels. Note, also, the widespread laking of red cells and the presence of numerous leucocytes outside the vessels. x 150.

Fig. 15. Photomicrograph from the chamber illustrated in Fig. 14 showing an extravascular collection of transparent globules. Two blood vessels can be seen (out of focus) in the background. x 150.

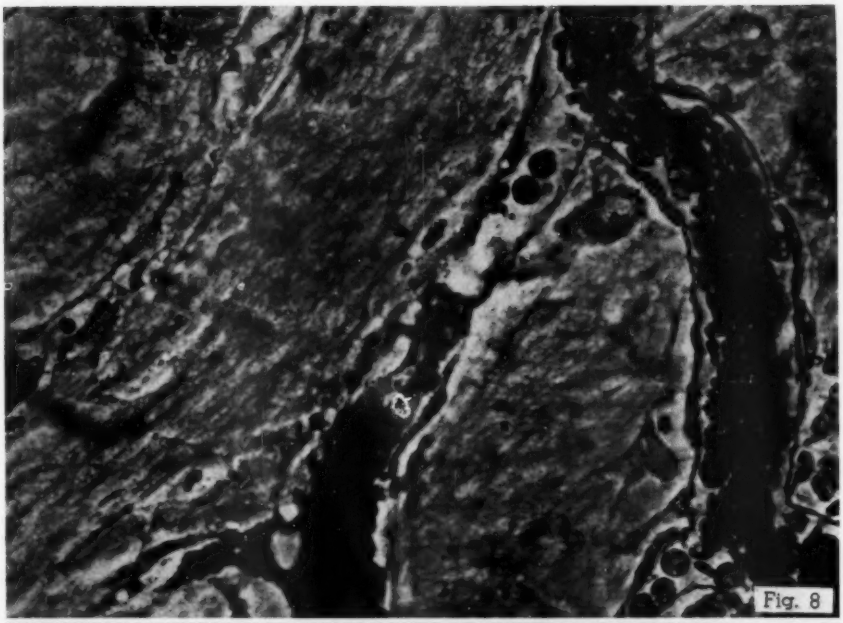
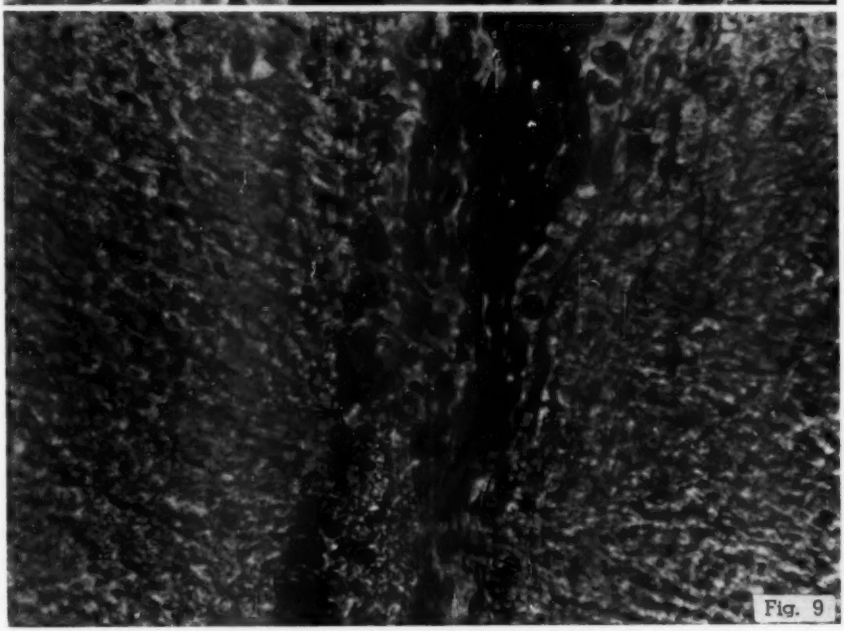


Fig. 8



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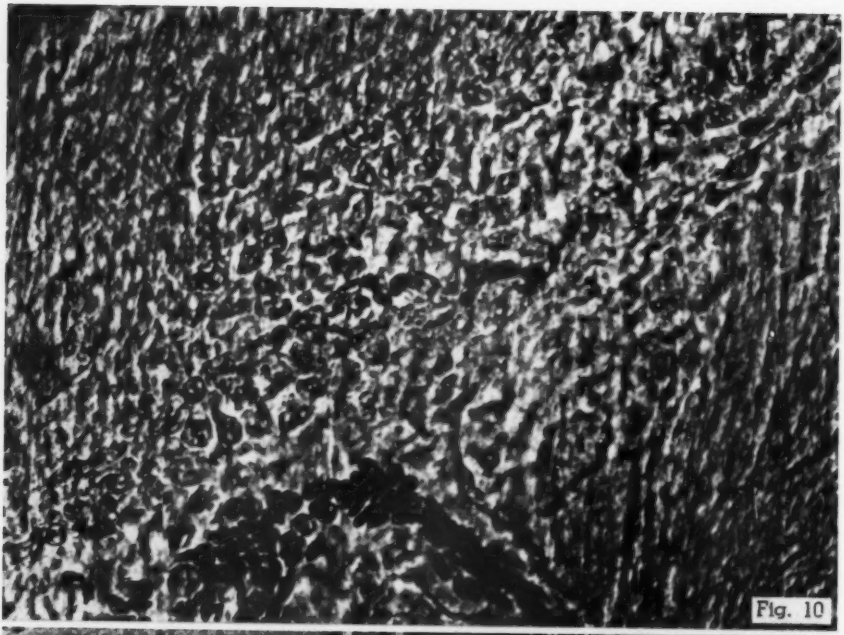

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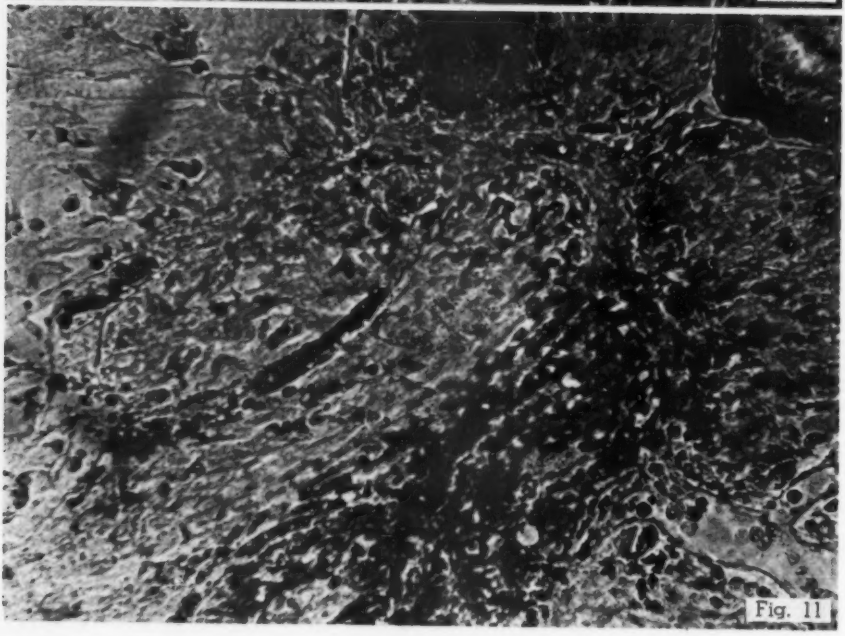

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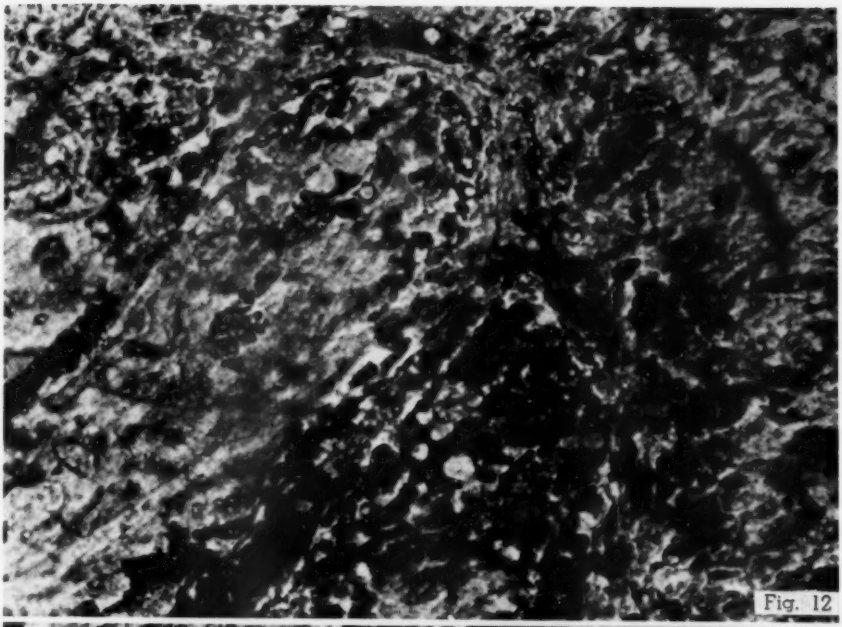

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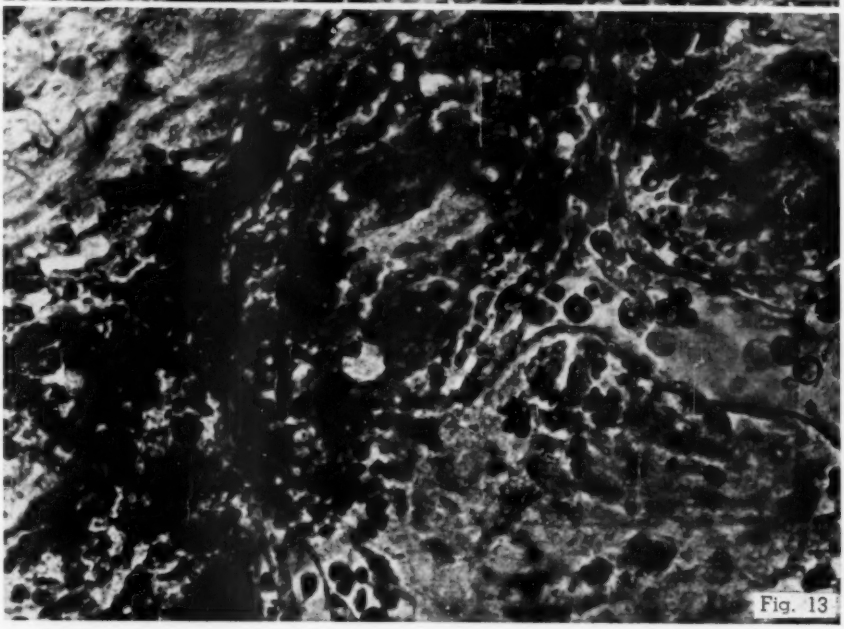

Fig. 13

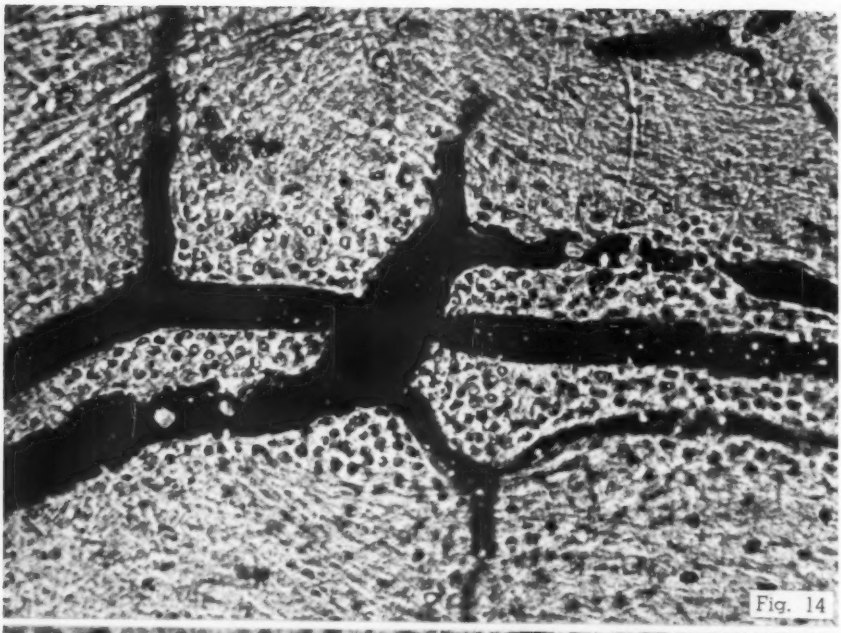

Fig. 14

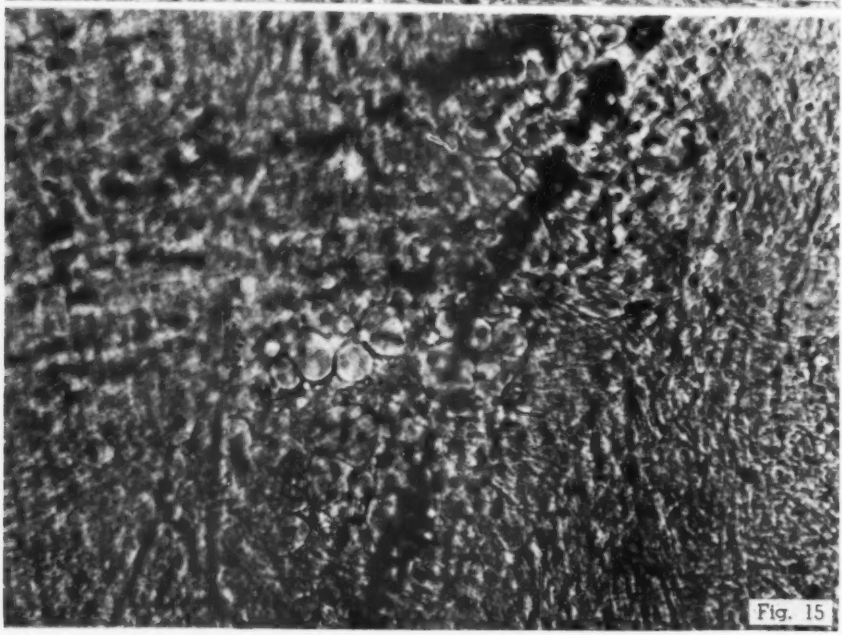

Fig. 15

after injury, and presented the following picture (Figs. 11, 12 and 13). Blood no longer flowed through the damaged vessels. Parts of some vessels were contracted and devoid of contents while adjacent segments contained coalesced red cells together with some clear globular material. The side walls of vessels were no longer clearly defined. Outside the vessels the predominant feature was the accumulation of intact and haemolysed red cells together with small masses of clear globular material similar in appearance to that seen previously within the lumen of the injured vessels. Macrophages were increased, but leucocytes were few in number. However, a number of leucocytes were present in the uninjured vessel segments immediately adjacent to the injured vessels; most of these had a rounded form and several contained a small round clear vacuole (Fig. 13). It thus appeared that the extent and severity of injury increased with the passage of time.

Post-mortem changes in ear-chambers.

Observations made on tissue in the ear-chamber of a rabbit which had been dead 24 hours disclosed widespread intra- and extravascular globules similar to those already described (Figs. 14 and 15). It was noted also that all red cells had coalesced into a homogeneous red mass and that many leucocytes were situated outside blood vessels.

DISCUSSION.

The present technique using high frequency current makes possible the production of minute areas of injury localized to any desired site within an ear-chamber. Further, because the mica cover remains unperforated, it facilitates the long-term study of tissue injury in the absence of secondary bacterial infection. The technique has revealed some consequences of damage to connective tissue which are obscured by less discriminatory methods of injury. In particular, it has been possible to show the development of transparent material in the form of "blisters" at the surface of injured endothelial cells with its subsequent separation to form free floating globules. In addition, it has made practicable the demonstration of delayed, peripherally extending signs of tissue damage which developed subsequent to the initial changes.

Whilst blisters and globular bodies were a constant finding in experimentally injured blood vessels, they were never observed in uninjured vessels. Further, as these structures formed in the ear-chamber of a dead rabbit, in the absence of heat injury, it is clear that they are not specific for heat injury. Observations of blisters situated on the endothelial cell surface, steadily increasing in size, strongly suggest that they are formed by the injured endothelial cell. The subsequent detachment of blisters to form free floating globules indicates that the numerous intravascular globules, whose development was not observed directly, also take their origin from blisters.

As yet there is no evidence of the possible pathological consequences of the free floating globular bodies. Nevertheless, in a severe burn the numbers present in the circulation might be very large and the possibility arises that their embolic lodgment in small vessels on a large scale might have serious consequences. It may be suggested that if this were the case, such structures would have been observed frequently in sectional material. However, although similar blisters and globules have been reported in electron micrographs (Schulz, 1956; Bergstrand and Bucht, 1956), they do not survive the tissue processing procedures used in paraffin embedding and therefore have not been observed in routine histopathological sections.

Allison, Smith and Wood (1955) described "globular bodies of unknown identity and significance" within and outside heat-injured blood vessels in rabbit ear-chambers. There is no doubt that these structures correspond to the "globules" described in the present work. Their attempts to stain these structures with fat colorants were unsuccessful and whilst their nature remained obscure, it was considered that they might correspond to the "agglomerates of protein" described by Kabat and Levine (1942) and Moritz *et al.* (1947) in the heated blood of animals.

Zweifach (1954) described in injured blood vessels ". . . a massive elaboration into the blood stream of a gelatinous exudate or precipitate, in which the blood cells become enmeshed". While this may be descriptive of the transparent globular material, it could apply equally well to the formation of platelet thrombus. However, it is likely that both phenomena occurred together in Zweifach's experiments.

The changes observed in red cells fall into two distinct categories. First the merging and coalescing of red cells into an apparently homogeneous red mass, usually described as "laking" and known to follow many types of injury. Second, the fragmentation of red cells, observed previously by Shen, Ham and Fleming (1943) and by Sevitt (1957) in the peripheral blood of patients suffering from burns. In the present experiments this phenomenon was immediately apparent following thermal injury, that is, within 15 seconds (the time taken to observe with the high power lens), and further, was not observed to occur subsequently. This suggests that the change may be specific for thermal injury; however, until detailed investigations employing other forms of injury are carried out the position remains uncertain.

In the present experiments vascular dilatation usually failed to occur following injury. This may have been due to the lack of innervation of arterioles of immature connective tissue, with consequent absence of an axon reflex. This interpretation is supported by the observation that arterioles in these chambers did not exhibit the periodic contractions characteristic of innervated arterioles in the rabbit. At all events, a quiescent circulation favoured the detailed study of blister and globule formation because the blisters then attained a greater

size before separating and, when separated, remained in the damaged vessel for a longer time. When rates of flow were high, blisters still appeared but were swept away at an earlier stage of development as smaller globules. Furthermore, they were more difficult to see in rapidly flowing blood, and those not swept from the endothelial surface were largely obscured by developing thrombus.

It is of interest that the signs of damage progressively increased in severity and extent for a considerable period after the initial injury. The existence of delayed injury developing as a consequence of the initial injury is difficult to establish mainly because the technique employed must exclude other possible causes of *continuing* injury; in particular, secondary bacterial infection. This latter appears to be excluded in the present experiments because the mica cover which prevents infection by air-borne bacteria remains unperforated.

One possible explanation of the delayed signs of injury is that the small area initially showing signs of damage is surrounded by a more peripheral zone in which the cells, although injured, do not develop visible structural changes for some hours. Another possibility is that following any injury, chemical factors, which are liberated from the injured cells, secondarily damage the surrounding cells.

Delayed tissue changes following injury have been reported by a number of investigators. Delayed and peripherally extending oedema following thermal injury has been shown in the guinea pig by Sevitt (1958) and confirmed for the rat by Spector and Willoughby (1958). Employing the rabbit ear-chamber, direct observations suggestive of delayed peripherally extending increased capillary permeability were made on thermally injured tissue by Allison, Smith and Wood (1955).

Although there is not necessarily any causal relationship between the delayed increase of capillary permeability and the present findings of delayed endothelial cell damage, the times of their occurrence coincide. It is possible that diffusible chemical factors liberated by injured cells secondarily damage adjacent endothelial cells, and that increased capillary permeability is one of the consequences of this cell damage. Finally, the possibility remains that both diffusible chemical factors and latent cell damage together contribute to the delayed signs of tissue injury. It is apparent that the elucidation of these problems must await further experiment.

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TISSUE INJURY BY HIGH FREQUENCY ELECTRIC CURRENT: OBSERVATIONS WITH THE SANDISON-CLARK EAR-CHAMBER

II. INJURY TO SMALL GROUPS OF CELLS

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SUMMARY.

Injury by high frequency electric current to small groups of cells within the Sandison-Clark rabbit ear-chamber has demonstrated the *in vivo* development of blister-like structures on injured cells and the subsequent separation of these to form free floating globular bodies. It is concluded that this process is responsible for the appearance of transparent globules within injured blood vessels and that it corresponds to a similar phenomenon frequently observed on cells *in vitro*.

Further consequences of cellular injury were the very rapid formation of cytoplasmic vacuoles by some cells and the active migration of granular leucocytes to the site of injury. Leucocyte accumulation under these conditions strongly suggests a chemotactic effect by products of injured cells.

INTRODUCTION.

Transparent blister-like structures, previously described within damaged blood vessels in the rabbit ear-chamber (Buckley, 1960a) are herein shown to be a cellular response to injury. This has been established by localizing high frequency current injury to selected avascular areas, some completely acellular, containing only connective tissue fibres with ground substance and others containing connective tissue cells and leucocytes. Blisters formed only in the cellular areas, and detailed study showed their cellular origin. In addition, the present work indicates that products of aseptically injured cells have a chemotactic influence on granular leucocytes in the living rabbit.

Materials and Methods.

These have been outlined in the companion paper (Buckley, 1960a), and described in greater detail elsewhere (Buckley, 1960b; Buckley and Semkiw, 1959).

RESULTS.

Injury to avascular, acellular areas of connective tissue. To explore the possibility that the transparent material might be formed as a result of heating intercellular fluid or connective tissue fibres, high frequency current was applied to a number of avascular, acellular areas. Although the connective tissue fibres became more transparent, in no instance did globular material subsequently appear.

Injury to small group of cells. For clarity, one representative example is described in detail. The changes to be described occurred in cells at the edge of the central table of a rabbit ear-chamber which, because of the extreme thinness of the space, had not become vascularized (Fig. 1). The cells, fibroblasts (F), leucocytes (L) and unidentified cells (U) are shown as they were 3½ minutes before damage (Fig. 2). For descriptive purposes each cell has, besides the letter signifying cell type, a number. To produce injury the electrode was placed on the mica cover at the point marked X and the high frequency current applied. The first high power photomicrograph, taken 1½ minutes after injury, already showed a number of changes (Fig. 3). Granular leucocytes (probably polymorphonuclear leucocytes) L1-5 which had been moving about in amoeboid fashion became immobilized and remained "frozen" in the shape they held at the time of injury. Fibroblasts were changed in appearance, their regular curved outlines being considerably distorted, and their cytoplasm mottled; in addition, F1 and F2 showed intracytoplasmic vacuoles. The nuclei appeared more refractile and had lost their regular curves, that of F2, for instance, being roughly triangular. The three nucleoli of fibroblast F2 fused into one irregularly shaped body. The mitosis of fibroblast F8 was arrested in telophase, individual chromosomes appearing more refractile and distinct. Apart from an alteration in position, unidentified cells U1-3 showed no immediate change.

Within three minutes of injury two small blister-like structures appeared on fibroblast F2 followed two minutes later by a single one on leucocyte L3 and several on fibroblast F8 (Fig. 4). When first seen, these blister-like structures were either hemispherical or ovoid with a very small area of contact with the cell surface. They gradually increased in size, maintaining the same general shape and showing an increasing area of contact with the cell surface. These blisters were transparent, devoid of internal structure and their boundary with the surrounding extracellular fluid was very sharply demarcated.

After 25 minutes a blister was seen on leucocyte L2, followed after 30 minutes by one on unidentified cell U3. However, some cells, for example leucocytes L1 and L3, although immobilized and therefore presumably injured, did not at any time show blisters.

When blisters were formed, they gradually increased in size (Figs. 5 and 6) and on one occasion adjacent blisters coalesced to form a large blister (Figs.

7 and 8). The volume of blister material was, in relation to the parent cell volume, very large. In the case of fibroblast F8 it was observed (Fig. 7) that the blister area was approximately three times that of the cell. After variable periods of time all blisters separated from their cells of origin and drifted away as free floating globular bodies (Fig. 9). Some of these drifting bodies slowly changed shape, sometimes appearing ovoid and sometimes bean-shaped (Figs. 10, 11 and 12).

A slowly progressive increase in number and size of intracytoplasmic vacuoles occurred in the fibroblasts. This was most obvious in fibroblasts F1 and F2, but could also be seen in the cytoplasm of F8 which was mottled in the early stages of the experiment (Fig. 4).

At the time of injury there were nine granular leucocytes in the high power field (Fig. 2), half an hour later there were 13 (Fig. 8), after one hour 46 (Fig. 9), and two hours later 70 (Fig. 16). Apart from the nine leucocytes immobilized in the area at the time of injury, they were all actively motile. On many occasions leucocytes came into close contact with one or other of the free floating globules (Figs. 11-16). They moved over this material, closely applying themselves to its surface by veil-like extensions of cytoplasm (Figs. 12-16). On one occasion a leucocyte ingested some of the clear globular material (Figs. 13 and 14); others, although not observed in the process of ingestion, were subsequently noted to contain a large very clear cytoplasmic vacuole (Figs. 10, 15, 16 and 17). The picture was, however, a constantly varying one, leucocytes continuously moving on and around the damaged cells and transparent globules. At one stage, they were concentrated at the very edge of the table top immediately adjacent to the damaged cells (Figs. 12-14).

Twenty-two hours after injury considerable changes had occurred (Fig. 18). No blisters remained on cells and only a few small globules were left in the high power field. Some cells had become detached from the mica cover, and while maintaining their original external shape, had lost most of their internal structure, taking on a ground-glass appearance. Adjacent fibroblasts showed accentuation of the earlier changes, considerable shrinkage, and further distortion of external form. The intracytoplasmic vacuoles, most noticeable immediately adjacent to the nucleus, were somewhat larger and more numerous. Of a total of 44 leucocytes in the field, seven had taken on the ground-glass appearance, six were in rounded form and 31 showed amoeboid movement. Two leucocytes contained a small round clear vacuole.

DISCUSSION.

The development of blisters on the surface of cells damaged by high frequency electric current, together with their non-occurrence in similarly treated avascular, acellular areas, leaves little doubt that these structures are formed by the injured cells. Blister formation with subsequent detachment to form free

EXPLANATION OF PLATES.

The time before or after injury is shown in the bottom left-hand corner of each photomicrograph.

Fig. 1. Low power view of avascular area at the edge of the central table of a rabbit ear-chamber 24 minutes before injury. The "target" cells lay to the left within the rectangle while to the right was a considerable number of leucocytes, some appearing as dark round structures, others as irregularly shaped bodies packed together at the edge of the central table. x 375.

Fig. 2. Higher power view of "target" cells 3½ minutes before injury showing a number of fibroblasts (F), motile granular leucocytes (L), and unidentified cells (U). The letters together with the numbers identify each cell for descriptive purposes. Note mitosis of fibroblast F8. x 700.

Fig. 3. Target cells 1½ minutes after injury showing immobilized leucocytes, distortion of nuclear and cytoplasmic outlines of fibroblasts together with vacuoles within cells F1 and F2. x 700.

Fig. 4. Five minutes following injury vacuoles in fibroblasts F1 and F2 became more clearly defined. Two blisters appeared on fibroblast F2 and three small blisters began to form on fibroblast F8. A small blister can be seen on the right side of leucocyte L3. x 700.

Fig. 5. Ten minutes following injury the three blisters on the upper half of fibroblast F8 had increased in size causing lateral displacement of leucocyte L4. Two new blisters had formed in the space between fibroblasts F7 and F8. x 700.

Fig. 6. Fifteen minutes after injury the lower half of fibroblast F8 had developed a new blister on its left side while those already formed had become larger. x 700.

Fig. 7. Twenty-four minutes following injury, blisters had formed on unidentified cell U3 and leucocyte L2 whilst those on the upper part of fibroblast F8 had further enlarged then coalesced to form one large blister. Intracytoplasmic vacuoles in fibroblast F2 had increased in size. Note that the leucocytes in the field at the time of injury, except L7, had not subsequently changed shape. x 700.

Fig. 8. Twenty-nine minutes after injury the blister on the upper part of fibroblast F8 had become smaller, having coalesced with, then separated from, an adjacent blister to which it contributed some of its substance. x 700.

Fig. 9. One hour following injury blisters had separated from the cells of origin to form free floating globules. Considerable numbers of amoeboid granular leucocytes had migrated to the area of injury. Note also numerous small particles of undetermined nature (see arrows). x 700.

Fig. 10. Seventy-three minutes following injury changes had occurred in the form and position of leucocytes which indicate active amoeboid movement. Note two clear vacuoles within the leucocyte adjacent to fibroblast F8. x 700.

24' Before injury

Fig. 1

3.5' Before injury

Fig. 2

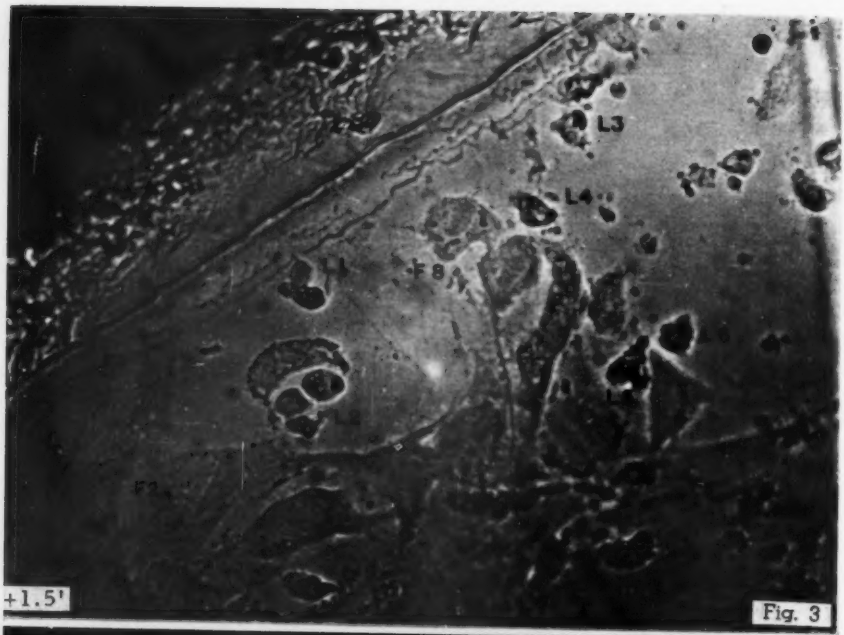


Fig. 3

Fig. 4

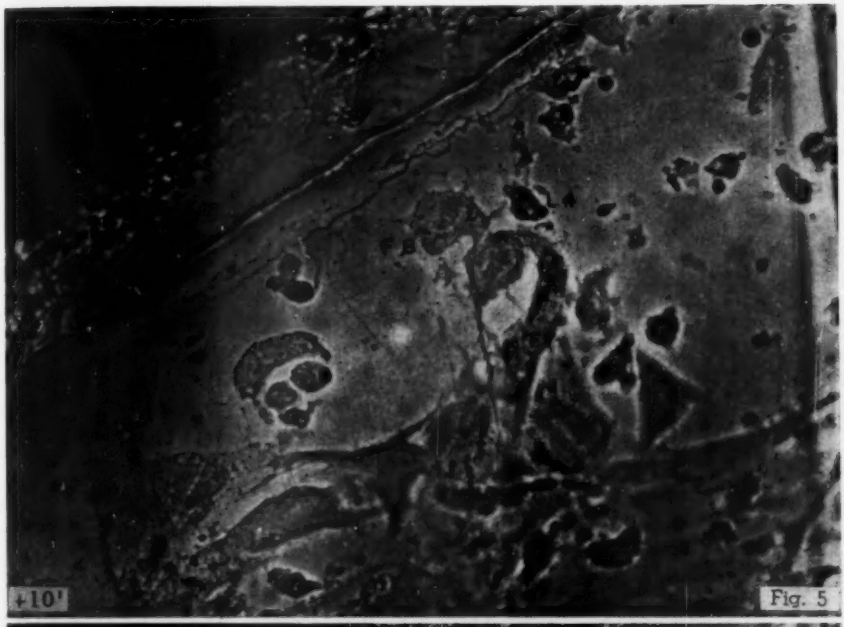


Fig. 5

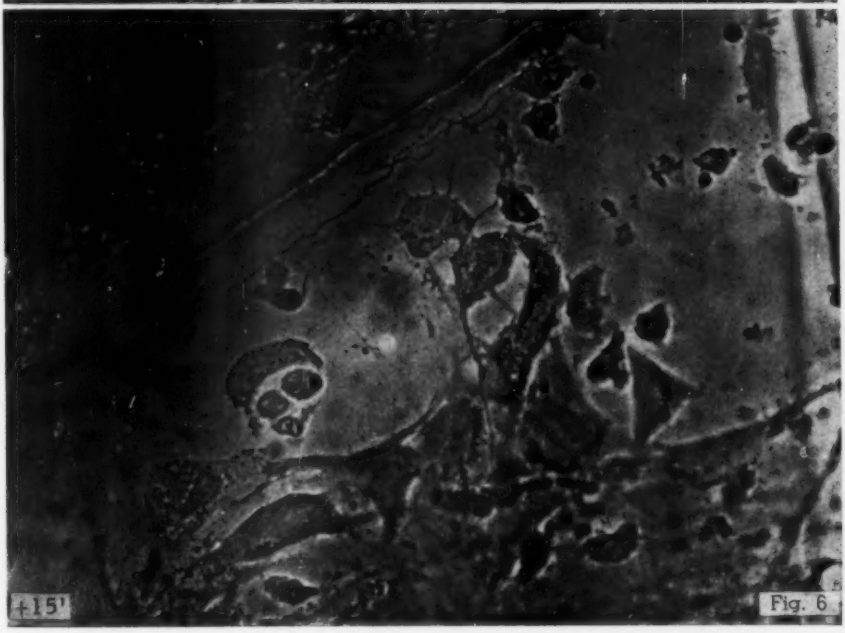


Fig. 6

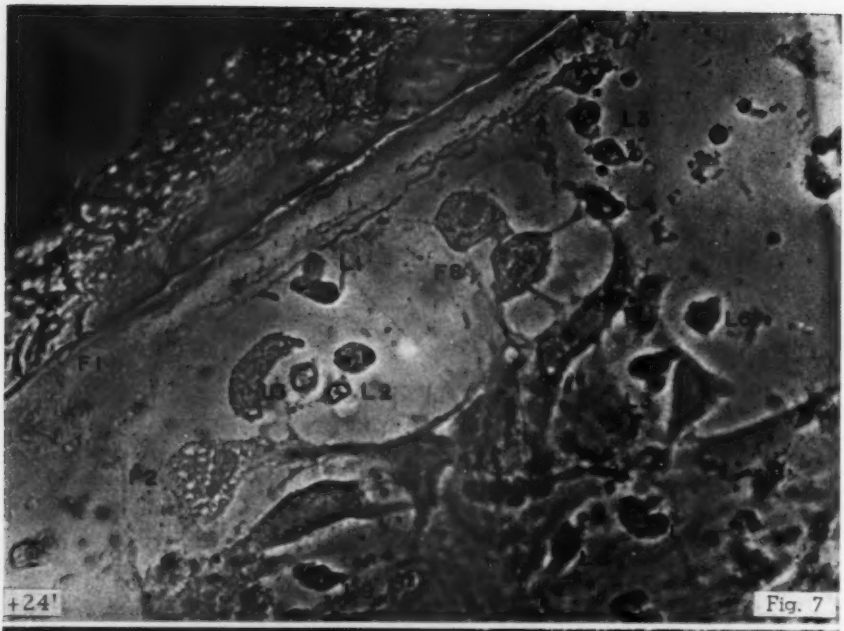


Fig. 7

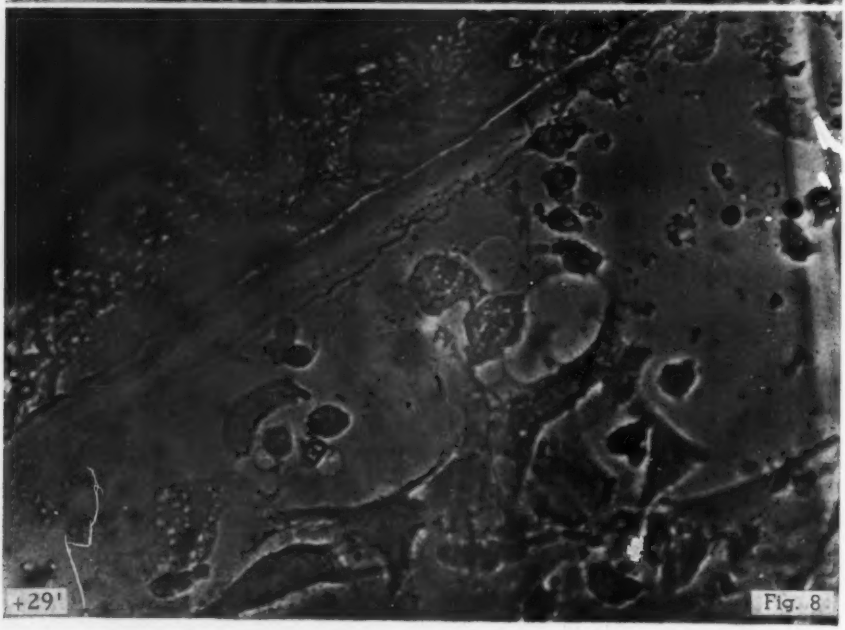


Fig. 8

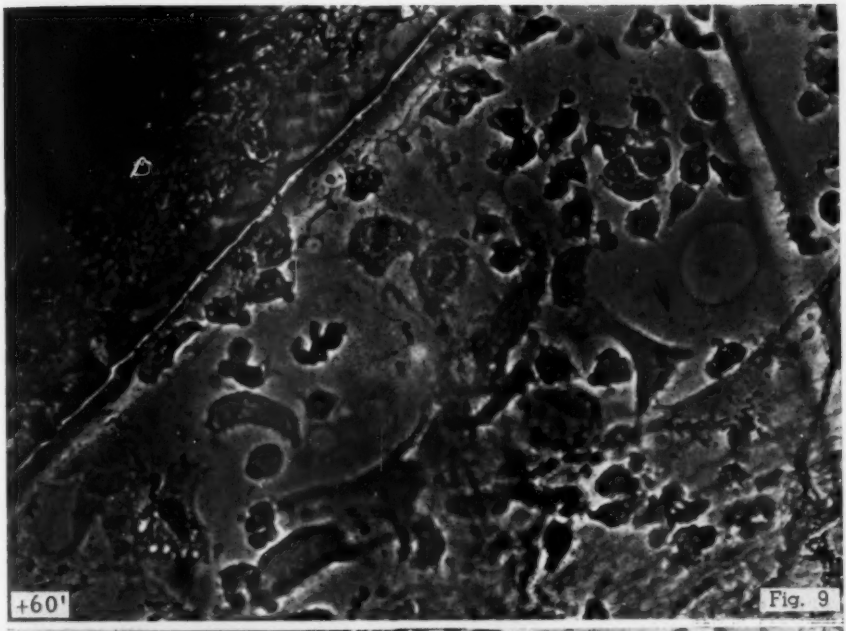


Fig. 9

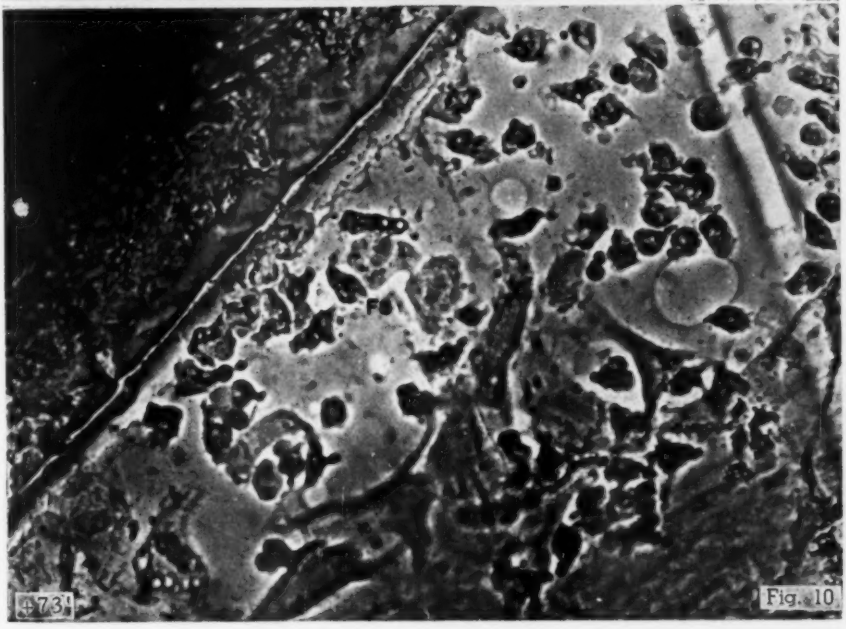


Fig. 10

EXPLANATION OF PLATES.

Fig. 11. Seventy-seven minutes after injury there were 66 actively motile leucocytes in the field. Leucocytes can be seen in close contact with the surfaces of six free floating globules (see arrows). x 700.

Fig. 12. Eighty-five minutes following injury veil-like extensions of cytoplasm from two leucocytes were seen closely applied to the surface of the large globule located just to the right of the centre of the field (see arrows). Note several leucocytes moving over fibroblast F8. x 700.

Fig. 13. Ninety-three minutes after injury one leucocyte just to left of centre of the field was observed in the initial stages of phagocytosing globular material (see arrow). x 700.

Fig. 14. Seven minutes later, one hundred minutes after injury, the leucocyte shown in Fig. 13 was seen to have ingested some of the globular material (see arrow). Note the very close contact between another leucocyte and fibroblast F2. x 700.

Fig. 15. One hundred and sixteen minutes after injury three leucocytes each contained a relatively large clear vacuole (see arrows). The leucocytes previously concentrated near the edge of the central table had dispersed. x 700.

Fig. 16. One hundred and eighteen minutes after injury several leucocytes were grouped around the large globule on the right: between this area and the table edge, close contact between a single leucocyte and a small globule could be seen in profile (see arrow). Vacuoles within fibroblasts F1 and F2 had become much smaller. x 700.

Fig. 17. One hundred and forty minutes following injury a temporary aggregate of leucocytes was seen to the right of the field while the large globule previously surrounded by leucocytes (see arrow) had drifted further to the right. x 700.

Fig. 18. Twenty-two hours after injury some of the injured cells had become detached from the mica cover and taken on a ground-glass appearance with loss of internal structure. Other injured cells were grossly shrunken and distorted, containing numerous small intracytoplasmic vacuoles, while both globules and leucocytes were reduced in number. x 700.

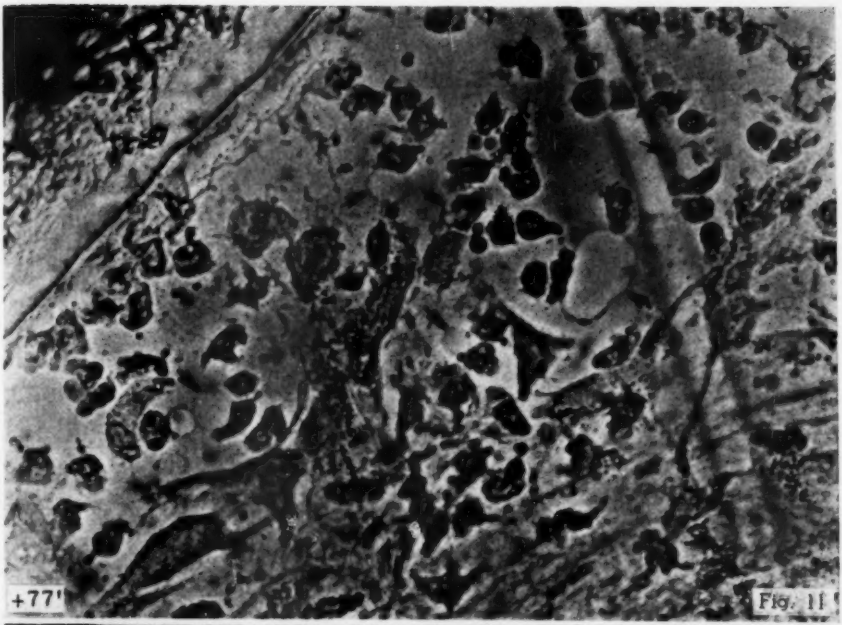


Fig. 11

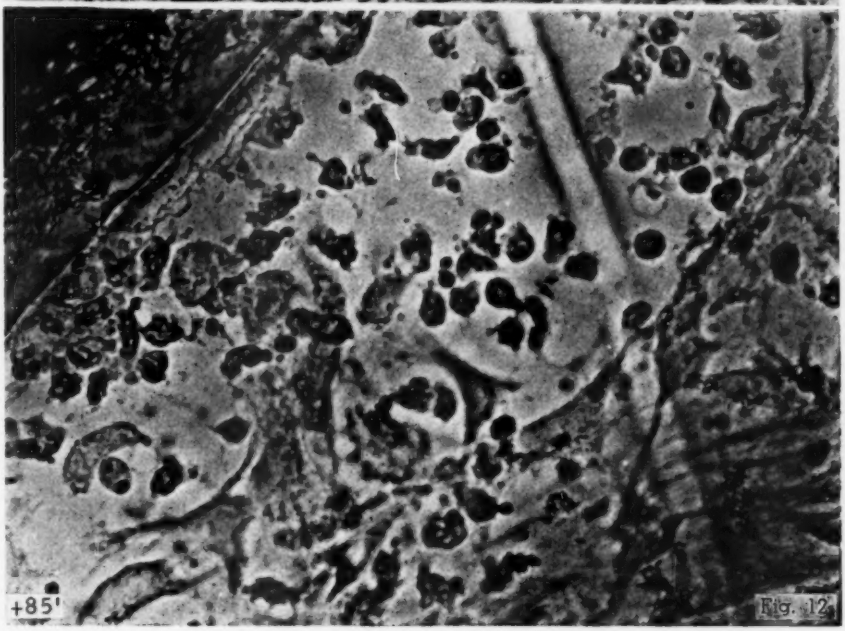


Fig. 12

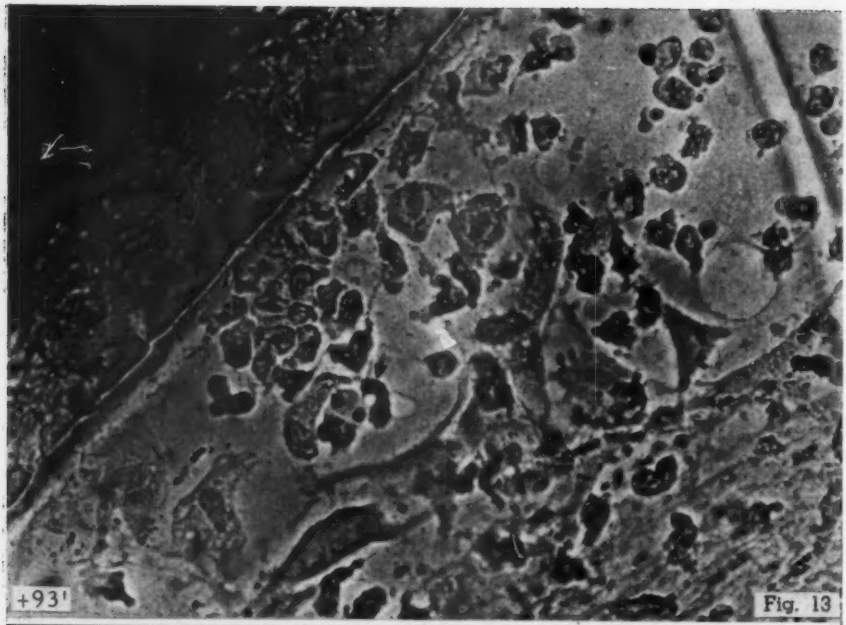


Fig. 13

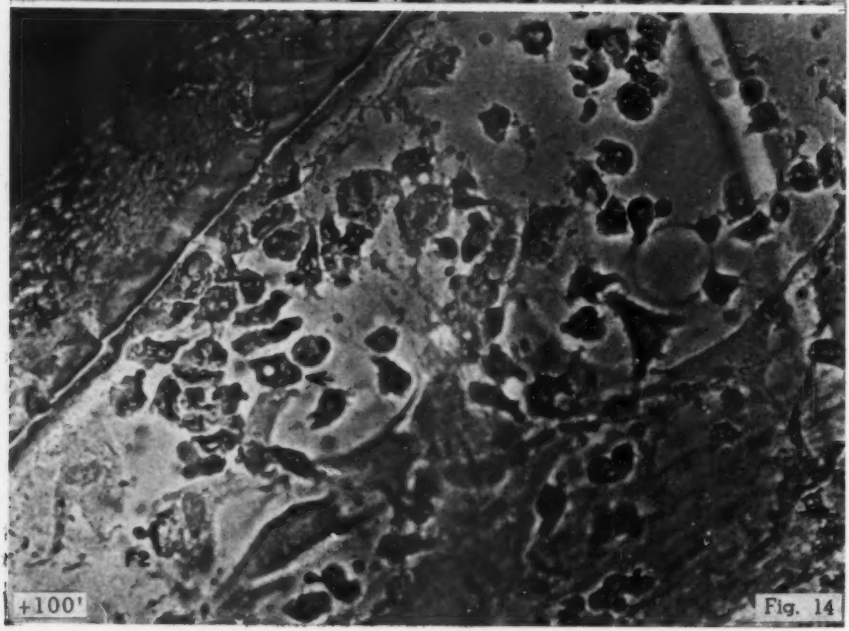
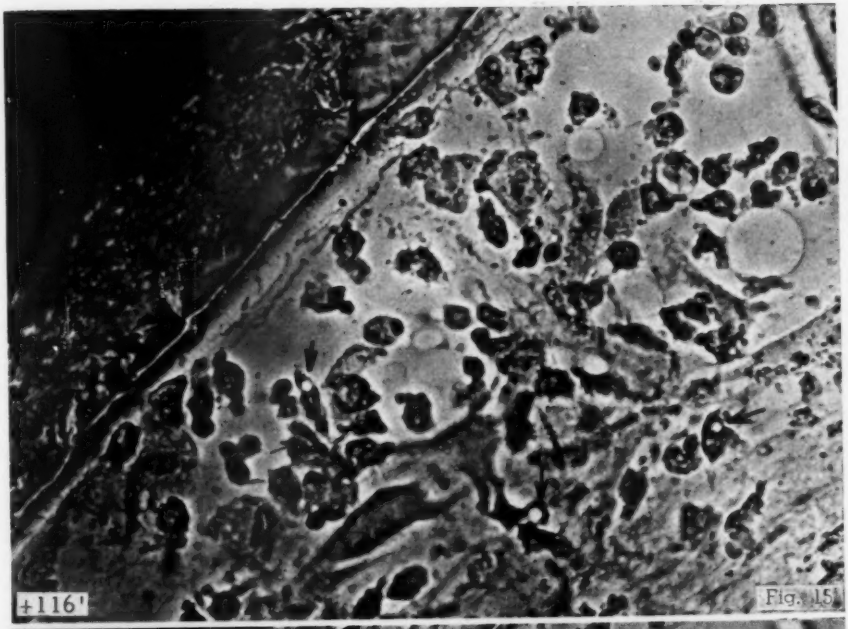


Fig. 14



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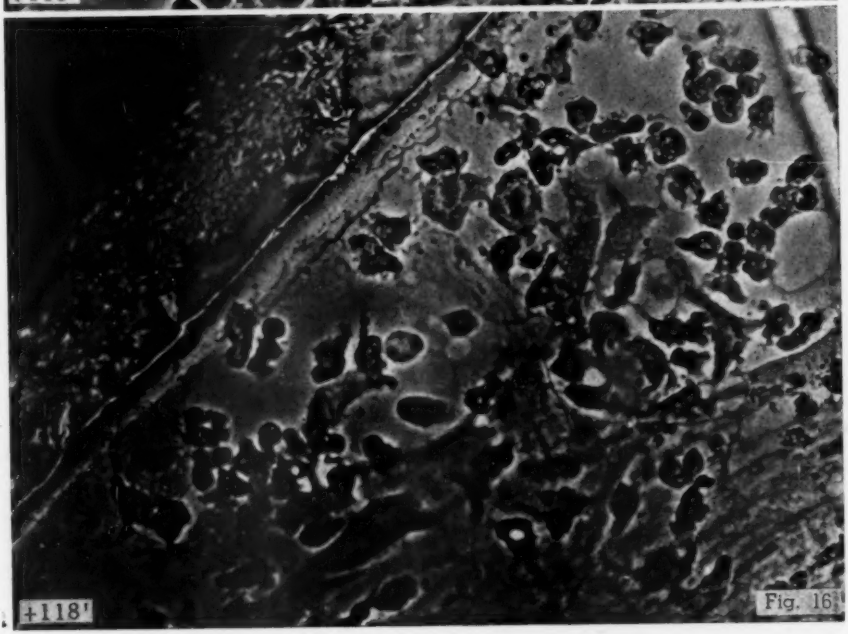

Fig. 16.

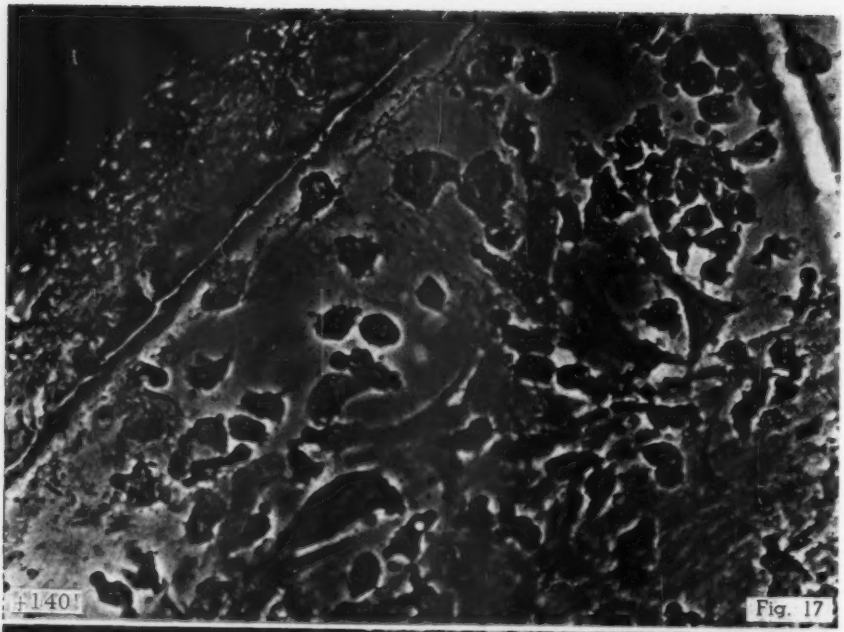

Fig. 17

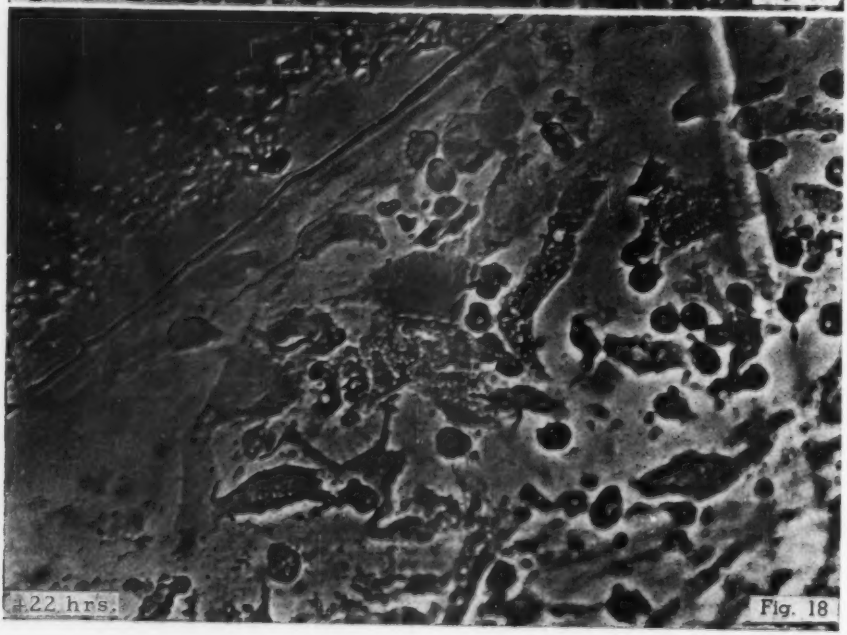

Fig. 18

floating globules, previously seen in relation to the endothelial cells of blood vessels (Buckley, 1960a), has been observed with leucocytes and fibroblasts. It is possible that all types of cells may respond in this way to thermal injury. However, failure of some presumably damaged cells to develop blisters indicates that this change is not an invariable consequence of injury. Subsequent work (Buckley, to be published) shows that blisters are formed by cell phospholipids taking up water from the surrounding medium.

The phenomenon of blister formation on the surface of cells has not been described previously *in vivo*, but has been frequently observed in *in vitro* cell studies. For instance, some protozoa, namely Paramoecium and Stentor (Chambers, 1924) and Vorticella (Bessis, 1949), have been noted to form blisters following injury. Again, since the development of tissue culture techniques they have been described repeatedly (Hogue, 1919; Lewis, 1923; Latta and Kutsakis, 1957). Zollinger (1948a, b, c, d) made a detailed study of blister formation using frog, mouse and rabbit cells in suspension. In addition he noted blister-like structures in a frozen section of unfixed human kidney. He concluded that one could not be sure that blisters formed in the intact living animal (or human) in either health or disease, and that probably they corresponded to cloudy swelling in the living organism. Bessis (1956), studying human bone marrow cells kept between slide and coverslip, described blister formation as one of a number of modes of cell death. Further, he showed blister formation to result from cell compression and from contact with various "physiological" salt solutions.

Although blister formation occurred at an early stage following injury, an earlier sign of cell damage was the appearance of intracytoplasmic vacuoles. The mechanism by which these form is not known, but their rapid formation excludes the biochemical synthesis of vacuole contents. Another mechanism by which vacuoles form is that of pinocytosis (Bessis, 1956). Whilst this was not observed in the present study using the light microscope, evidence from electron microscopic studies (Palade, 1955, 1956; Parks and Chiquoine, 1956) indicates that pinocytosis involving the endoplasmic reticulum may be a common occurrence. However, the great rapidity of vacuole formation makes this mechanism seem an unlikely one, and the solution of the problem must await further experiment.

The accumulation of granular leucocytes in the region of injured cells may be a true example of chemotaxis by aseptically injured cells. While the chemotactic influence of certain bacteria and some other substances is now well established by *in vitro* methods, there is as yet little direct evidence for chemotaxis *in vivo*. As pointed out by Harris (1954) there has been no convincing demonstration of chemotaxis by injured cells under sterile conditions. Moreover, he emphasized that in any *in vivo* study of this phenomenon it is necessary to exclude increased capillary permeability, passive transport of leucocytes, leuco-

cyte immobilization following chance contact with the test material, and bacterial contamination since any of these could give a false positive result. In the present work it appears that none of these complicating factors was operating. The damaged cells were in an avascular area, and, although vessels lay in the connective tissue adjacent to the central table, the migrating leucocytes came from the surrounding avascular areas on the central table and not from the adjoining vascular regions. Leucocytes at all times progressed by their active amoeboid movement, never by passive transfer; they were not immobilized following contact with the injured cells. Finally, since the method of injury did not involve puncturing the mica cover of the chamber, the probability of bacterial infection is very remote. Thus the present observations strongly support the hypothesis that, in the rabbit, products of aseptically damaged tissue are chemotactic for granular leucocytes. This *in vivo* finding is at variance with the *in vitro* work of Harris (1953a, b), but in accord with the observations of Allison, Smith and Wood (1955) that actively motile leucocytes accumulate at the centre of thermally injured connective tissue within the rabbit ear-chamber.

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AUGMENTATION BY TRIIODOTHYRONINE OF SOME OF THE EFFECTS OF HYDROCORTISONE IN MAN

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SUMMARY.

There has been recent interest in the interaction between thyroid and adrenal hormones arising out of studies of the metabolic changes associated with stress in man.

A comparison has been made of the metabolic effect of hydrocortisone (given by continuous infusion in 100 mg. and 200 mg. dosage) with the combination of hydrocortisone (in the same dosage) and a single intravenous injection of 0·5 mg. of L-triiodothyronine. An infusion of normal saline on another occasion acted as a control. Observations were carried out in six healthy medical student volunteers, each infusion being maintained for an eight-hour period of observation.

Hydrocortisone alone did not raise the metabolic rate or alter the non-protein respiratory quotient. The metabolic rate was increased by the combination with triiodothyronine, the rise observed being similar to that previously determined from a similar dosage of triiodothyronine alone.

Triiodothyronine augmented the increase in blood glucose and fall in blood eosinophils observed with hydrocortisone alone.

The decrease in plasma inorganic phosphate between the second and sixth hours of observation with hydrocortisone was altered to a significant increase after the fourth hour when triiodothyronine was also given.

Changes in plasma cholesterol levels were unexpected. While there was no change with hydrocortisone alone, the combination with triiodothyronine produced an increase which was significant at two and eight hours.

Following the combination there were no consistent differences in the urinary excretion rates of total nitrogen, sodium, potassium and inorganic phosphate when compared with levels following separate administration.

These results indicate that the simultaneous administration of triiodothyronine augments some, but not all, of the effects of hydrocortisone.

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INTRODUCTION.

There has been recent investigation of the possibility of interaction between thyroid and adrenal hormones in man (Brooks *et al.*, 1957; Fremont Smith *et al.*, 1957). This has arisen mainly as a result of studies of the metabolic disturbances associated with surgical trauma (Moore, 1957) from which indirect evidence has been adduced for the importance of thyroid as well as of adrenal hormones (Goldenberg *et al.*, 1954). Further indirect evidence has been adduced from observations of the similar metabolic changes associated with emotionally stressful life experiences in man (Hetzel *et al.*, 1956a). These findings led to a study which demonstrated an alteration of the metabolic response to adrenaline by pretreatment with triiodothyronine and hydrocortisone (Hetzel *et al.*, 1958a).

In the light of all these considerations, it was thought of interest to determine whether the effect of hydrocortisone itself could also be influenced by simultaneous administration of triiodothyronine.

METHODS.

Observations were made on six healthy medical student volunteers on each of three occasions after fasting overnight. Constancy of food intake on the previous evening was not always possible. Fluctuations in the initial values for blood glucose, plasma cholesterol and plasma inorganic phosphate in individual subjects may reflect this variation. Each treatment was given on each occasion in random fashion. An infusion of normal saline at the rate of 1 ml. per minute was given over a period of eight hours each day beginning between 8 a.m. and 9 a.m. It was given alone as a control; and to it was added hydrocortisone as the free alcohol (100 mg. for four subjects, O.C., Q.P., R.G., S.A., and 200 mg. for the other two subjects, P.R. and T.A.) on another occasion; while on the other day a single injection of 0·5 mg. L-triiodothyronine[3] was also given into the tube in addition to the hydrocortisone at the beginning of the infusion.

Samples of blood were collected before the infusion and at two hourly intervals afterwards for eight hours. Determinations of blood glucose using the method of Nelson (1944), plasma cholesterol (Zak *et al.*, 1954), plasma inorganic phosphate (Taussky and Shorr, 1953) and eosinophil count (Pilot, 1950) were carried out on these samples.

The metabolic rate and gaseous exchange were also determined at two hourly intervals over the eight-hour period beginning one hour after starting the infusion. This was done using the Hartmann-Braun basal metabolic rate indicator which provides direct reading of O_2 deficit and CO_2 elimination from a hot wire and Wheatstone Bridge circuit. In each case observations were made at one minute intervals for ten minutes and the mean values taken. The non-protein respiratory quotient (NPRQ) was also calculated.

Urine samples were collected on arrival at the laboratory and at hourly intervals throughout the eight-hour period of infusion. Determinations of sodium and potassium excretion using the EEL flame photometer, inorganic phosphate (Taussky and Shorr, 1953), total nitrogen (micro Kjeldahl with Nesslerisation) and creatinine (Bonsnes and Taussky, 1945) were carried out.

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[3] Kindly supplied by Glaxo Laboratories Ltd., Greenford, Middlesex.

RESULTS.

Effect on metabolic rate. The results of determinations of metabolic rate are shown in Table 1. Under control conditions there was a consistent tendency

TABLE 1.

Effect of hydrocortisone (F) and a combination of hydrocortisone and T3 (T3+F) on metabolic rate compared with control (S).

(Calories per square metre per hour.)

Subjects	Treatment*	Time in hours			
		1	3	5	7
O.C.	T3+F	31·7	40·5	44·8	43·9
	F	41·6	40·3	43·1	42·8
	S	39·4	39·9	42·2	39·8
P.R.	T3+2F	45·6	46·8	47·7	50·9
	2F	44·7	44·3	46·9	48·8
	S	52·0	37·3	39·7	49·0
Q.P.	T3+F	41·7	40·4	46·1	44·7
	F	39·7	40·4	42·7	44·0
	S	38·8	36·7	40·2	41·6
R.G.	T3+F	41·6	42·5	46·0	46·4
	F	40·6	40·9	44·4	44·0
	S	41·8	41·6	41·7	45·6
S.A.	T3+F	45·2	49·5	47·3	54·3
	F	48·4	49·4	48·8	51·5
	S	43·0	42·5	44·7	46·6
T.A.	T3+2F	45·2	46·3	49·8	52·6
	2F	44·5	44·4	46·8	49·0
	S	40·6	44·6	45·6	47·2
Mean	T3+F	41·8	44·3	47·0	48·8
	F	43·2	43·4	45·5	46·8
	S	42·6	40·5	42·4	45·0

Trend analysis reveals a significant rise with T3+F ($P<0·05$) but no change with F alone compared with S.

* T3=0·5 mg. triiodothyronine; F=100 mg. hydrocortisone; 2F=200 mg. hydrocortisone; S=control (saline).

for the metabolic rate to rise. This has been noted previously in these studies (Hetzel *et al.*, 1957) and has been attributed to increasing restlessness of the subjects associated with forced recumbency.

Trend analysis revealed no significant difference between hydrocortisone and control, but a significant increase with triiodothyronine (T3) and hydrocortisone (F) together ($P<0·05$).

No consistent effects on NPRQ were noted following hydrocortisone either alone or in combination with T3.

Effect on blood glucose. The data on blood glucose are shown in Table 2. A slight fall was noted under control conditions; a significant increase occurred with hydrocortisone alone ($P < 0{\cdot}01$) at six and eight hours, while a further significant increment occurred by eight hours when T3 was given with hydrocortisone ($P < 0{\cdot}01$). This was evident with both 100 mg. and 200 mg. dosages of hydrocortisone (Table 2).

TABLE 2.

Effect of hydrocortisone (F) and a combination of hydrocortisone and T3 (T3+F) on blood glucose mg. p.c.

Subjects	Treatment*	Time in hours				
		0	2	4	6	8
O.C.	T3+F	102	102	104	101	102
	F	100	97	95	105	105
	S	106	95	96	94	87
P.R.	T3+2F	87	102	106	115	120
	2F	100	103	93	106	110
	S	90	100	97	100	98
Q.P.	T3+F	105	103	107	110	127
	F	100	101	103	103	101
	S	97	93	94	96	95
R.G.	T3+F	73	95	100	103	100
	F	85	85	85	100	93
	S	80	85	85	80	70
S.A.	T3+F	90	105	110	115	117
	F	98	90	104	102	105
	S	93	97	100	95	87
T.A.	T3+2F	86	86	86	97	102
	2F	80	97	103	95	90
	S	90	95	80	83	83
Mean	T3+F	91	99	102	107	111
	F	94	96	97	102	101
	S	93	94	92	91	87
P**	(T3+F)	—	—	—	—	<0·01
	(F)	—	—	—	<0·01	<0·01

* T3=0·5 mg. triiodothyronine; F=100 mg. hydrocortisone; 2F=200 mg. hydrocortisone; S=control (saline).

P** probability using "t" test where—
(T3+F) indicates comparison of T3+F with F.
(F) indicates comparison of F with S.

Effect on plasma inorganic phosphate. The results are shown in Table 3. The level of plasma inorganic phosphate remained steady over the eight-hour period under control conditions, but a significant fall was noted from two to

six hours with hydrocortisone alone. The administration of triiodothyronine and hydrocortisone was followed by a significant increase at four, six and eight hours ($P < 0{\cdot}01$).

TABLE 3.

Effect of hydrocortisone (F) and a combination of hydrocortisone and T3 (T3+F) on plasma inorganic phosphate mg. p.c.

Subjects	Treatment*	Time in hours				
		0	2	4	6	8
O.C.	T3+F	3·5	2·9	2·7	2·7	3·0
	F	3·0	2·4	2·3	2·6	2·5
	S	3·0	2·7	3·2	2·7	3·3
P.R.	T3+2F	3·7	2·3	3·0	3·7	4·0
	2F	2·5	2·4	2·6	2·5	2·9
	S	3·4	3·0	2·7	3·2	3·3
Q.P.	T3+F	2·8	2·1	2·6	3·0	3·0
	F	2·8	2·6	2·2	2·4	2·5
	S	2·9	2·8	3·3	3·2	3·0
R.G.	T3+F	3·7	2·5	3·5	4·2	4·7
	F	3·9	3·4	2·8	3·3	3·8
	S	3·5	3·0	3·6	3·7	3·7
S.A.	T3+F	3·5	2·9	3·6	4·1	4·0
	F	3·1	3·2	3·4	3·4	3·4
	S	3·5	3·7	4·0	3·9	3·5
T.A.	T3+2F	3·4	3·3	3·2	3·8	3·5
	2F	3·4	3·1	2·9	2·9	3·4
	S	2·9	3·5	3·5	3·4	3·4
Mean	T3+F	3·4	2·7	3·1	3·6	3·7
	F	3·1	2·8	2·7	2·8	3·1
	S	3·2	3·1	3·4	3·3	3·3
P**	(T3+F)	<0·05	–	<0·01	<0·01	<0·01
	(F)	–	<0·05	<0·01	<0·01	–

* T3=0·5 mg. triiodothyronine; F=100 mg. hydrocortisone; 2F=200 mg. hydrocortisone; S=control (saline).

P** probability using "t" test where—
(T3+F) indicates comparison of T3+F with F.
(F) indicates comparison of F with S.

Effect on plasma cholesterol. The results shown in Table 4 reveal a steady level over the eight-hour period under control conditions with no significant difference following hydrocortisone. A significant increase occurred following triiodothyronine and hydrocortisone at two and eight hours ($P < 0{\cdot}01$) with less marked increases in between these times.

TABLE 4.

Effect of hydrocortisone (F) and hydrocortisone with T3 (T3+F) on plasma cholesterol mg. p.c.

Subjects	Treatment*	Time in hours				
		0	2	4	6	8
O.C.	T3+F	138	175	163	139	183
	F	155	160	150	130	143
	S	128	135	133	125	143
P.R.	T3+2F	145	155	138	160	158
	2F	138	143	120	150	143
	S	140	138	135	145	155
Q.P.	T3+F	158	193	195	189	205
	F	180	168	168	163	178
	S	168	175	165	178	172
R.G.	T3+F	178	168	155	165	163
	F	143	138	150	158	143
	S	143	143	143	150	143
S.A.	T3+F	155	150	153	150	178
	F	123	145	160	133	153
	S	170	183	165	178	165
T.A.	T3+2F	170	170	158	150	156
	2F	150	165	160	168	150
	S	150	143	150	150	155
Mean	T3+F	157	169	160	159	174
	F	148	153	151	150	152
	S	150	153	149	154	156
P**	(T3+F)	–	<0·01	–	–	<0·01
	(F)	–	–	–	–	–

* T3=0·5 mg. triiodothyronine; F=100 mg. hydrocortisone; 2F=200 mg. hydrocortisone; S=control (saline).

P** probability using "t" test where—

(T3+F) indicates comparison of T3+F with F.

(F) indicates comparison of F with S.

TABLE 5.

Effect of hydrocortisone (F) and a combination of hydrocortisone and T3 (T3+F) on total eosinophil count per cm.

Subjects	Treatment*	Time in hours			
		0	2	4	6
O.C.	T3+F	50	6	0	6
	F	39	6	11	0
	S	42	28	11	11
P.R.	T3+2F	10	17	17	0
	2F	17	11	17	6
	S	22	28	17	33
Q.P.	T3+F	139	72	111	0
	F	194	211	117	56
	S	183	106	161	100
R.G.	T3+F	39	44	0	6
	F	50	7	28	6
	S	33	61	83	67
S.A.	T3+F	89	33	11	0
	F	28	39	11	11
	S	106	22	111	80
T.A.	T3+2F	128	128	22	0
	2F	67	15	22	6
	S	117	89	61	122
Mean	T3+F	76	50	27	2
	F	66	48	34	14
	S	84	56	74	57
P**	(T3+F)	—	—	—	<0·01
	(F)	—	—	—	<0·01

* T3=0·5 mg. triiodothyronine; F=100 mg. hydrocortisone; 2F=200 mg. hydrocortisone; S=control (saline).

P** probability using "t" test where—
(T3+F) indicates comparison of T3+F with F.
(F) indicates comparison of F with S.

Effect on blood eosinophils. The data shown in Table 5 reveal a slight fall under control conditions, a significant fall in six hours following hydrocortisone ($P < 0{\cdot}01$) and a further significant fall ($P < 0{\cdot}01$) when triiodothyronine was also given with the hydrocortisone.

Effects on urinary constituents. The effects were similar to those previously described following hydrocortisone and triiodothyronine alone (Hetzel *et al.*, 1957). There was no consistent difference in the excretion rates of total nitrogen, sodium or potassium following hydrocortisone when T3 was also given. The previously described phosphate diuresis (Hetzel *et al.*, 1957; Hetzel *et al.*, 1958b) was as evident following triiodothyronine and hydrocortisone, as following triiodothyronine alone.

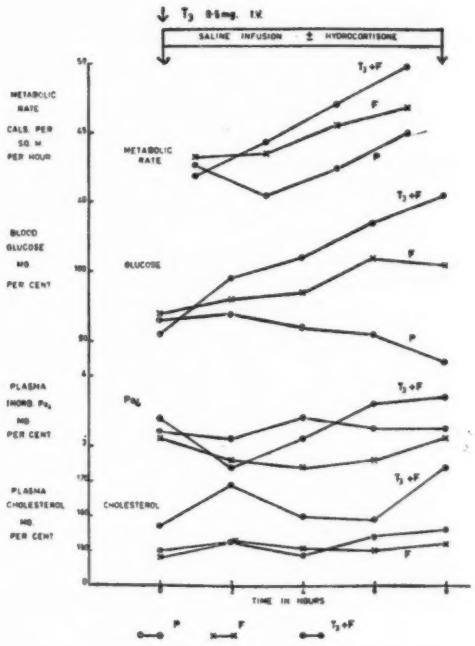

Fig. 1. Effect of hydrocortisone (F) and a combination of hydrocortisone and triiodothyronine (T3 + F) on metabolic rate, blood glucose, plasma inorganic phosphate and plasma cholesterol.

DISCUSSION.

The metabolic effects of hydrocortisone have been the subject of extensive study and the findings in this investigation generally confirm what has previously been reported (Hetzel *et al.*, 1957; Thorn *et al.*, 1953). There was no significant elevation of metabolic rate which is consistent with the findings of Hills *et al.* (1950), though a slight increase was observed in hypothyroid subjects by these investigators. No consistent changes in NPRQ were noted following hydrocortisone. A rise in blood glucose unaccompanied by increase in total nitrogen excretion has also been reported previously (Thorn *et al.*, 1953); this finding indicates that gluconeogenesis is not an important factor at this stage, though the evidence favours an action of adrenal cortical hormone on the liver rather than peripheral tissues (Thorn *et al.*, 1957; Altzuler *et al.*, 1957). A rise in serum cholesterol following hydrocortisone has previously been reported from this laboratory (Hetzel *et al.*, 1957) using a less specific method for determination of cholesterol (Zlatkis *et al.*, 1953), but no increase was demonstrated over the

eight-hour period of observation in this study. The fall in plasma inorganic phosphate following hydrocortisone has been previously reported and shown to be accompanied by a fall in urinary excretion (Hetzel *et al.*, 1957; Mills and Thomas, 1957). These observations suggest that the phosphate has moved into the cells presumably to offset an increase in cations (Hetzel *et al.*, 1957). By ten hours an increase in serum phosphate and an increase in urinary excretion occur (Hetzel *et al.*, 1957).

With the addition of triiodothyronine (0·5 mg. intravenously as a single dose) a significant increase in metabolic rate was apparent within eight hours as previously found with triiodothyronine alone (Hetzel *et al.*, 1957; Hetzel *et al.*, 1958b). This early increase has been shown to be accompanied by a rise in serum and urine inorganic PO_4, but not by any evidence of nitrogen breakdown (Hetzel *et al.*, 1957). Previous studies have shown that there is no significant stimulation of adrenal cortical secretion following triiodothyronine in 0·5 mg. dosage (Fremont Smith *et al.*, 1957; Hetzel *et al.*, 1957). Neither was a fall in eosinophil count observed following 0·5 mg. triiodothyronine alone over a period of six hours (Hetzel *et al.*, 1957).

It is of interest that following both hormones there was a significant further rise in blood glucose when compared with hydrocortisone alone. There was also a significant rise in plasma inorganic PO_4 following both hormones. These two changes could be linked in that a loss of phosphate from the cells might interfere with the phosphorylation of glucose and thus diminish glucose utilisation. There may also be an increase in glucose production from glycogen in the liver as suggested previously to account for the increase in blood glucose with adrenaline following pretreatment with triiodothyronine (Hetzel *et al.*, 1958a).

The rise in plasma cholesterol following both hormones was an unexpected finding in view of the fact that there was a tendency for the level to fall with T3 alone within ten hours in a previous study (Hetzel *et al.*, 1957). This is followed by a definite fall after twenty-four hours both in hypothyroid and normal subjects (Hetzel *et al.*, 1957; Asper *et al.*, 1953; Rawson *et al.*, 1953). There is evidence that the fall in plasma cholesterol following thyroid hormones is due to increased hepatic storage of cholesterol while in the rat biliary excretion of cholesterol is increased following thyroid administration (Byers *et al.*, 1952; Lipsky *et al.*, 1955; Rosenman *et al.*, 1951). Variable effects have been described following long term cortisone in man depending on the type of patient receiving it (Eder, 1958), while mobilisation of depot fat has been shown to occur following cortisone in rats (Levin and Farber, 1952). It is apparent that no definite conclusion can be drawn as to the mechanisms involved in the rise in plasma cholesterol observed here.

Some suggestions as to the mechanism by which thyroid hormones could enhance the effects of hydrocortisone may be made. There is evidence of an increase in disappearance rate of hydrocortisone in thyrotoxicosis (Levin and

Daughaday, 1955; Peterson, 1958) and this increase may be largely due to an increase in hepatic cell turnover of the hormone (Samuels *et al.*, 1957). The data reported here would suggest that the action of hydrocortisone on the liver cell is facilitated in some way by the thyroid hormone so that its effects on blood glucose and plasma cholesterol are augmented. This may occur by alterations in distribution between plasma and tissues as previously suggested (Samuels *et al.*, 1957). Another possibility is suggested by the recent demonstration of the effect of thyroid hormones on the morphology of liver mitochondria (Tapley, 1956). Further studies of the action of these hormones at the cellular level are required and are being carried out in this laboratory.

Augmentation by triiodothyronine of the effect of hydrocortisone on the total eosinophil count may be secondary to the increase in metabolic rate (Wintrobe, 1942). However, as already mentioned, no fall in the eosinophil count was observed following triiodothyronine alone when increase in metabolic rate occurred (Hetzel *et al.*, 1957).

These results are of interest in relation to the previously reported enhancement by triiodothyronine of the effect of A.C.T.H. in hypopituitarism (Brooks *et al.*, 1957). Increases in plasma hydrocortisone and 17-ketogenic steroid excretion were observed when triiodothyronine was given to two patients with hypopituitarism on a constant maintenance dosage of A.C.T.H. It was uncertain, therefore, whether the associated increase in fasting blood glucose, changes in sodium and potassium balance and fall in eosinophils were due to this increase in circulating adrenal cortical hormone or also to augmentation of its effect by triiodothyronine. The results of the present investigation indicate that augmentation of the effects on blood glucose, plasma cholesterol and eosinophil count does occur. However, augmentation of the effects on sodium and potassium excretion could not be demonstrated over the period of observation. Fremont Smith *et al.* (1957) in their longer term study in two normal subjects failed to demonstrate any difference in the effects of triiodothyronine or hydrocortisone on sodium and potassium (or nitrogen) balance whether given separately or together.

The significance of these findings in relation to the mechanisms involved in the metabolic changes associated with stress is uncertain. The results of this study, together with previous ones from this laboratory and elsewhere (Brooks *et al.*, 1957; Fremont Smith *et al.*, 1957; Hetzel *et al.*, 1957), indicate that a combination of thyroid and adrenal hormones will reproduce most of the features of the stress response. Moreover, the results of the present study indicate that the increase in the circulating levels of both these hormones which has been described under these conditions (Moore, 1957; Schwartz and Roberts, 1957; Hetzel *et al.*, 1955; Hetzel *et al.*, 1956b; Board *et al.*, 1956) may be associated with enhancement of some, though not all, of the effects of hydrocortisone. There is no support for the view which has been previously advanced (Goldenberg *et al.*, 1954) that these hormones are antagonistic in their effects.

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AN IMPROVED MICROBIOLOGICAL ASSAY FOR FORMIMINOGLUTAMIC ACID

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SUMMARY.

A microbiological assay for formiminoglutamic acid using *Lactobacillus arabinosus* as test organism is described. The method can be used to detect formiminoglutamic acid in urine when other growth-promoting substances such as glutamic acid and glutamine are present. By use of this method, the urinary excretion of formiminoglutamic acid can be regularly detected in folic acid-deficient rats.

INTRODUCTION.

The detection of formiminoglutamic acid in the urine appears to have clinical significance in folic acid-deficiency states. This substance is an intermediate product in the metabolism of histidine and requires tetrahydrofolic acid for further degradation (Tabor and Wyngarden, 1958). Formiminoglutamic acid has been detected in the urine of folic acid-deficient rats (Rabinowitz and Tabor, 1958), of patients being treated with folic acid antagonist drugs (Broquist, 1956; Hiatt, Goldstein and Tabor, 1958), and of patients suffering from megaloblastic anaemia due to folic acid deficiency (Broquist and Luhby, 1959; Spray and Witts, 1959).

Formiminoglutamic acid can be estimated by procedures involving enzymatic reactions, the end products being measured microbiologically (Silverman, Gardiner and Condit, 1958) or spectrophotometrically (Tabor and Wyngarden, 1958). Silverman, Gardiner and Bakerman (1952) assayed the glutamic acid formed from formiminoglutamic acid by heat, using *Lactobacillus arabinosus* as test organism. This is a relatively simple microbiological technique, but may give misleading results with urine, due to the presence of free glutamic acid and glutamine, both of which will promote growth of the test organism. The present paper describes a modified microbiological assay using *Lactobacillus arabinosus* which appears to be specific for formiminoglutamic acid.

METHODS AND MATERIALS.

Principle of the method.

Unheated urine may contain glutamic acid and glutamine, both of which stimulate growth of *Lactobacillus arabinosus*. Formiminoglutamic acid is microbiologically inactive, but is heat labile and is converted to glutamic acid on heating. Comparison of the growth-promoting activities of heated and unheated urine does not give a true reflection of the formiminoglutamic acid content of the unheated specimen, as glutamine is degraded to pyrrolidone carboxylate by heat. In the present method glutamic acid and glutamine are removed from the urine by allowing the organism to grow in assay medium containing unheated urine. When growth ceases, the organisms are removed by centrifugation, the urine-medium mixture is heated, cooled and re-inoculated. Further growth is then due to glutamic acid liberated from formiminoglutamic acid during the heating process.

Organism and media.

The test organism, *Lactobacillus arabinosus* 17-5 (A.T.C.C. 8014), was subcultured from inoculum broth (Difco 0963) into glutamic acid assay medium (Difco 0961) containing 20 μg./ml. glutamic acid. After 48 hours' growth at 37° C., one drop of the culture was transferred into assay medium containing 19 μg./ml. glutamic acid. Subculture was repeated every 48 hours into assay medium containing progressively lower concentrations of glutamic acid until the organism grew readily in assay medium containing 5 μg./ml. glutamic acid. The effect of varying concentrations of glutamic acid on the growth responses of the mutant strain and the standard strain of *Lactobacillus arabinosus* is shown in Fig. 1. The mutant strain of the organism was preserved in the dry state.

At weekly intervals, a dried culture was placed in a tube containing inoculum broth and incubated at 37° C. for 24 hours. Subculture was then made onto slopes of culture agar

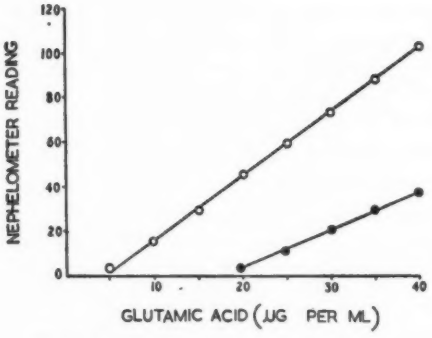

Fig. 1. Growth response to varying concentrations of glutamic acid of the mutant strain and the standard strain of *Lactobacillus arabinosus*. Mutant strain ○——○; standard strain ●——●.

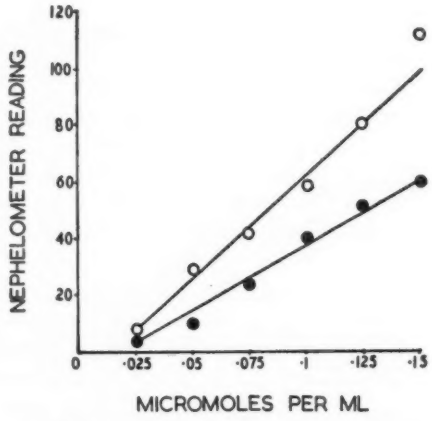

Fig. 2. Recovery of formiminoglutamic acid as glutamic acid. The lower line records the nephelometer readings given by varying concentrations of formiminoglutamic acid after conversion to glutamic acid by heat. The upper line records the readings given by the molar equivalents of glutamic acid.

(Difco 0962), and after incubation for 24 hours, the slopes were stored in the refrigerator at 4° C.

Two cultures are required for each test. The day before the test, subculture is made from a stock slope into a tube containing 10 ml. inoculum broth. After incubation at 37° C. for 24 hours the tube is centrifuged and the supernatant discarded. The organisms are washed once with 10 ml. of sterile normal saline, and then resuspended in 10 ml. of saline for use as the inoculum. On the first day of the test, the second culture is processed as described above.

Tests are carried out in 6in. × ⅝in. matched rimless tubes capped with oxoid aluminium caps. The tubes are washed with detergent (Aviscour), rinsed several times in running tap water and left overnight in 1 p.c. hydrochloric acid. They are rinsed several times in distilled water and twice autoclaved filled with distilled water (15 lb. pressure for 20 minutes). They are emptied, inverted and dried in the hot air oven.

Stock glutamic acid solution. This is made by dissolving 200 mg. L-glutamic acid hydrochloride in 100 ml. distilled water. The stock solution keeps for months if stored in the refrigerator.

First day of assay. Five ml. samples of urine are sterilized by passage through sintered glass sterilizing filters (porosity 5 on 3). Using an aseptic technique, one ml. aliquots are transferred to each of four tubes containing five ml. assay medium and four ml. distilled water previously sterilized by autoclaving at 15 lb. pressure (121° C.) for 10 minutes. It is important that the tubes be cooled before adding the urine samples, and at no time during the first stage of the test is the urine subjected to heat. Four control tubes are set up, each containing five ml. assay medium, four ml. distilled water and one ml. glutamic acid solution containing 200 μg. glutamic acid.

Each tube is inoculated with one drop of the standard inoculum and incubated at 37° C. for 20 hours.

Second day of assay. The tubes are removed from the incubator and centrifuged at 2000 r.p.m. for 30 minutes. The supernatants are carefully removed and placed in sterile 6in. × ⅝in. tubes. Three tubes from each series of four are autoclaved at 15 lb. pressure (121° C.) for 10 minutes. All tubes are inoculated with one drop of standard inoculum prepared from the second culture and incubated for 20 hours at 37° C. The unheated tube from each series of four serves as a control to show that no further growth occurs on re-inoculation of the unheated mixture.

Glutamic acid standard tubes. Five ml. stock glutamic acid solution are diluted to 100 ml. with distilled water, giving a glutamic acid concentration of 100 μg./ml. On the second day of the test, duplicate standard tubes containing varying quantities of glutamic acid are set up as shown in Table 1, autoclaved, cooled and inoculated along with the tubes containing the test mixtures.

TABLE 1.

Preparation of tubes for glutamic acid standard curve.

Tube number	Blank	1	2	3	4	5	6	7	8
Glutamic acid solution (100 μg./ml.) (mls.)	0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0
Distilled water (mls.)	5	4·5	4·0	3·5	3·0	2·5	2·0	1·5	1·0
Assay medium (mls.)	5	5	5	5	5	5	5	5	5
Final concentration of glutamic acid (μg./ml.)	0	5	10	15	20	25	30	35	40

Turbidity estimation. Growth is recorded with an EEL nephelometer. The reading of the blank standard tube is subtracted from the readings of all other standard tubes. A curve is plotted on linear graph paper of glutamic acid concentration against the corrected nephelometer readings. The readings of the re-inoculated unheated tubes are subtracted from the readings of the heated tubes. The resulting turbidity value is converted into glutamic acid concentration from reference to the standard curve.

RESULTS AND DISCUSSION.

It is most unusual to obtain growth in the unheated tubes after the second inoculation, indicating that glutamine and free glutamic acid have been utilised by the organisms after the first inoculation. Growth in the heated tubes is due to glutamic acid released from formiminoglutamic acid during the autoclaving process. Typical growth patterns given by solutions in distilled water of glutamine, glutamic acid hydrochloride and barium formiminoglutamate[1] are indicated in Table 2.

TABLE 2.

Growth patterns of solutions of glutamine, glutamic acid hydrochloride and barium formiminoglutamate.

	First inoculation	Second inoculation	
		Unheated	Heated
Glutamine	+	−	−
Glutamic acid HCl	+	−	−
Ba formiminoglutamate	−	−	+
Glutamine, glutamic acid HCl and Ba formiminoglutamate	+	−	+

The quantitative accuracy of the method was tested by adding known quantities of barium formiminoglutamate to tubes containing water and assay medium. The tubes were autoclaved and inoculated, and growth was compared with that occurring in tubes containing known quantities of glutamic acid hydrochloride. The results are shown in Fig. 2 where quantities of both barium formiminoglutamate and glutamic acid hydrochloride are expressed in micromoles. The recovery of formiminoglutamic acid as glutamic acid averaged 66 p.c. of the expected value, assuming that complete conversion had been achieved. These calculations have been based on Tabor's conclusion that the molecular weight of the material supplied to us is 280.

[1] Barium formiminoglutamate was kindly supplied by Dr. Herbert Tabor, National Institutes of Health, Bethesda, Maryland, U.S.A.

Using the method described in this paper we have been able to detect regularly the excretion of formiminoglutamic acid in the urine of rats fed a diet deficient in folic acid and containing succinylsulphathiazole.

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THE METABOLISM OF ISONIAZID AND OTHER HYDRAZIDES BY *MYCOBACTERIA*

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SUMMARY.

Mycobacterium tuberculosis (B.C.G.) metabolised isoniazid, a hydrazone of isoniazid and other hydrazides. An isoniazid resistant culture also metabolised isoniazid. It is suggested that the degradation of hydrazides is a detoxication mechanism and not directly related to the mode of action of isoniazid.

It is further suggested that cultures resistant to isoniazid metabolise isoniazid as fast as they take it up, while sensitive strains bind isoniazid more rapidly than they can metabolise it.

INTRODUCTION.

In a previous paper (Youatt, 1958a) the uptake of isoniazid by *Mycobacterium tuberculosis* (B.C.G.) was described. It was suggested then that the "metabolic" type of uptake might involve a process in which the isoniazid was changed. Paper chromatography of the supernatants and of extracts of cells after exposure to isoniazid have shown the presence of *iso*nicotinic acid. The formation of *iso*nicotinic acid from isoniazid has been detected in aqueous solution (Wenzel, Netter and Kistner, 1957), in the presence of hemin (Albert and Rees, 1955), in human urine (Cuthbertson and Ireland, 1953), and also in the presence of isoniazid sensitive and resistant *Mycobacteria* (Boone, Strang and Rogers, 1956). No experimental data were given by Boone *et al.* (1956), so it was not known whether the appearance of *iso*nicotinic acid was attributable to the organisms or was due to chemical breakdown. The present paper is concerned with the metabolism of isoniazid and other hydrazides by *Mycobacteria*.

METHODS.

Cultures. Details have been published previously (Youatt, 1958a). The organisms were grown on a modified Sauton medium for 7-10 days at 37° C. The surface growth was harvested and worked to a paste with a spatula on filter paper. The cells were washed and suspended in phosphate buffer, pH 7.

*Chemical estimations. Iso*nicotinic acid was estimated by the method of Rubin, Drekter, Scheiner and Ritter (1952). As crystalline cyanogen bromide was not available the reagent was prepared freshly when required by the addition of ice-cold sodium cyanide to ice-cold saturated bromine water. No protein precipitant was required. The samples consisted of

clear supernatants from the cells. Standards were included with all estimations. Colours were read with an Evelyn colorimeter.

Isoniazid was estimated with picryl chloride. Most of the difficulties encountered in the clinical estimation of isoniazid are due to the presence of protein. In the present system clear supernatants were used and a simpler method was possible. This was based on the method described by Cuthbertson and Ireland (1953) for the development of chromatograms. Samples were made up to 6 ml. with distilled water and 0·2 ml. of 0·3 p.c. picryl chloride and 0·1 ml. of 0·1M NaOH were added.

The same colour reaction was given by other hydrazides. Hydrazones were estimated as isoniazid after acid hydrolysis. This was readily carried out by boiling the samples for a few seconds at pH 2. The colour was not given by compounds which did not have the $NH\text{-}NH_2$ group intact. For example, no colour was produced with acetyl isoniazid, 1-*iso*-nicotinyl-1-methyl-hydrazine dihydrochloride or with 1-2 bis (*iso*nicotinyl) hydrazine.

In all experiments cell suspensions without isoniazid were incubated and sampled to supply blanks. These showed no colour with either reagent. Isoniazid concentrations were always sufficiently high to permit dilution of the samples which were then centrifuged, filtered and stored frozen until the end of the experiment when all the samples were estimated together.

RESULTS.

The metabolism of hydrazides. Suspensions of isoniazid sensitive and resistant B.C.G. in 0·07M phosphate buffer pH 7 were shaken in air with added hydrazides. Samples were taken for the estimation of hydrazide and, where possible, of the corresponding acid. The method for the estimation of *iso*nicotinic acid was also used for the estimation of nicotinic acid. Killed suspensions for controls were obtained by autoclaving at 118° C. for 10 minutes. Chemical

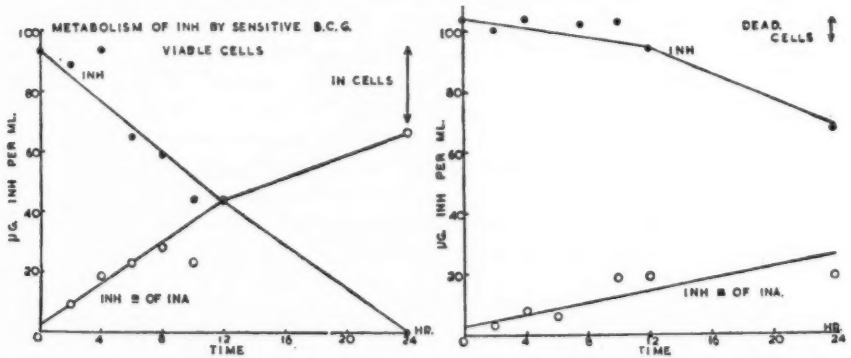

Fig. 1. *The metabolism of isoniazid by sensitive B.C.G.* Isoniazid sensitive B.C.G. suspensions in phosphate buffer pH 7 were shaken in air at 35° C. with radioactive isoniazid. Samples were taken at various times for the estimation of isoniazid and *iso*nicotinic acid in the supernatants and at 24 hours the radioactive content of the cells was also determined in order to calculate how much of the isoniazid was bound in the cells. The radioactive content of the cells and the *iso*nicotinic acid found were expressed as their equivalent of isoniazid. Dead cells were autoclaved for 10 minutes at 118° C.

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sterilising agents such as mercuric chloride and formaldehyde interfered with the chemical estimations.

Fig. 1 shows the breakdown of isoniazid by living and dead suspensions of isoniazid sensitive B.C.G. The amount of isoniazid or its products retained in the cells was determined from the radioactive count made on the cells after washing. From the known dry weight of cells per ml. of suspension the loss from the supernatants to the cells was calculated and expressed as μg. per ml. of isoniazid. To permit a direct comparison, the *iso*nicotinic acid produced was expressed as the equivalent concentration of isoniazid.

Fig. 2 shows a similar experiment with resistant B.C.G. Here the amount of radioactive material held in the cells is similar to that bound by killed sensitive organisms (Youatt, 1958a).

These results show a slower but quite measurable rate of breakdown in the presence of killed cells. In phosphate buffer pH 7 there was no detectable loss of hydrazides after shaking in air at 35° C. for 24 hours. It appeared probable that the slow breakdown of isoniazid by killed cells was due to a chemical reaction. Similar results have been obtained in preliminary experiments with cell-free extracts. Breakdown still occurred in extracts after boiling. This reaction also took place after dialysis and in the presence of o-phenanthroline, 8-hydroxyquinoline, and versene. It is not, therefore, due to a simple metal catalysis.

The rate of uptake and of oxidation of isoniazid varied from one batch of cells to another, depending presumably on the percentage of viable cells present. Thus, although comparison of Figs. 1 and 2 suggests that resistant cells oxidise isoniazid at a slower rate than sensitive cells do, Table 1 shows an experiment in which the rates were approximately equal. Over a number of experiments

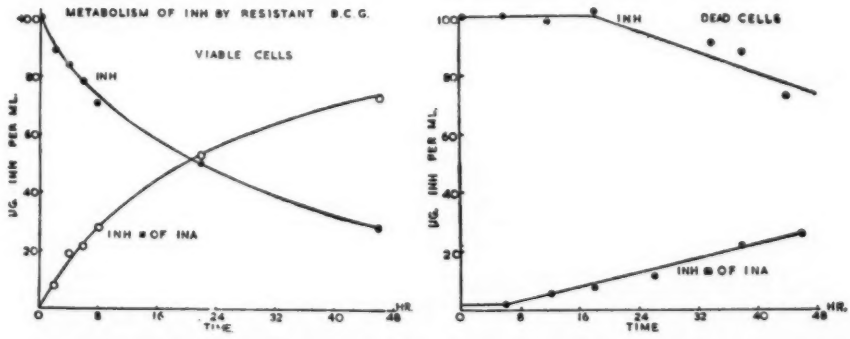

Fig. 2. *Metabolism of isoniazid by resistant B.C.G.* Isoniazid resistant B.C.G. suspension in phosphate buffer was shaken in air at 35° C. with isoniazid. Samples were taken for the estimation of isoniazid and *iso*nicotinic acid in the supernatants. The *iso*nicotinic acid produced was expressed as the equivalent concentration of isoniazid. Dead cells were autoclaved at 118° C. for 10 minutes.

the rates of oxidation by sensitive and resistant suspensions were not found to be appreciably different. However, the rates of uptake differed greatly between sensitive and resistant cells. In a series of eight experiments with different suspensions of sensitive and resistant B.C.G. the average rates of uptake with 20 μg./ml. isoniazid for two hours at 35° C. were 117 and 17 μg. isoniazid per gm. dry weight of cells for sensitive and resistant cells respectively.

The disappearance of other hydrazides was also followed by chemical estimation of the hydrazides with picryl chloride. Benzoic acid hydrazide concentrations were also followed by the purple colour produced with sodium pentacyanoaminoferroate. The rates of breakdown of the hydrazides in the presence of sensitive and resistant organisms are shown in Table 1.

TABLE 1.

Metabolism of hydrazides.

Sensitive and resistant B.C.G. suspended in phosphate buffer pH 7 were shaken in air at 35° C. with isoniazid (INH), nicotinic acid hydrazide (NH), benzoic acid hydrazide (BH) and picolinic acid hydrazide (PH) of initial concentrations of 50 μg./ml. Samples were taken at 0, 2, 4, 6 and 8 hours for the estimation of residual hydrazides. The rates quoted were calculated from the slope of the best straight line. Killed cells were autoclaved at 118° C. for 10 minutes.

Cell suspensions	Rate of breakdown μg./ml./hr.			
	INH	NH	PH	BH
Sensitive B.C.G. (viable)	4·5	2·0	3·3	3·4
Sensitive B.C.G. (killed)	2·2	1·3	1·1	1·5
Resistant B.C.G. (viable)	3·8	1·3	1·2	2·0
Resistant B.C.G. (killed)	0·6	0·3	0·8	0·5

Inhibition of the metabolism of isoniazid. The uptake of isoniazid required the presence of oxygen and was inhibited by sodium cyanide and sodium azide (Youatt, 1958a). The breakdown of isoniazid was similarly affected as shown in Table 2.

TABLE 2.

Inhibition of isoniazid breakdown.

Isoniazid sensitive B.C.G. suspended in phosphate buffer pH 7 was shaken in air at 35° C. for 16 hours with 100 μg./ml. isoniazid. Isoniazid (INH) and *iso*nicotinic acid (INA) were estimated by the methods described in the text.

Experimental conditions	μg./ml. INH lost	μg./ml. INA formed
Control	43	39
NaN_3 $M \times 10^{-3}$	10	12
NaN_3 $M \times 10^{-4}$	24	17
NaCN $M \times 10^{-3}$	7	0
NaCN $M \times 10^{-4}$	24	16
2-4 dinitrophenol $5M \times 10^{-4}$	11	
N_2 atmosphere	10	9

In the presence of 2-4 dinitrophenol much of the isoniazid was bound in acid hydrolysable form. Hydrazone formation by isoniazid would account for this observation and also, perhaps, for the initial stimulation of isoniazid uptake in the presence of 2-4 dinitrophenol (Youatt, 1958a). It is not known whether the 2-4 dinitrophenol is itself metabolised by the organism or whether it causes an accumulation of keto compounds in the cell suspensions.

Metabolism of a hydrazone of isoniazid. When a suspension of isoniazid sensitive B.C.G. was shaken in air at 35° C. with verazide (the hydrazone of isoniazid and veratric aldehyde) the hydrazone gradually disappeared, *iso*nicotinic acid appeared but no free isoniazid was detected in the supernatants. The rate of breakdown of the hydrazone was slower than that of isoniazid by the same suspension. The hydrazone is taken up rapidly. If the breakdown to *iso*nicotinic acid involves the intermediate formation of isoniazid, this must be the rate determining step.

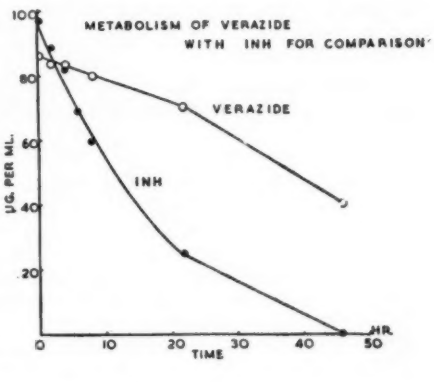

Fig. 3. *The metabolism of verazide.* Isoniazid sensitive suspensions in phosphate buffer pH 7 were shaken in air with isoniazid and verazide. The concentrations of verazide are quoted as their equivalent of isoniazid. Verazide concentrations were determined with picryl chloride after acid hydrolysis.

DISCUSSION.

Mycobacterium tuberculosis (B.C.G.) metabolised isoniazid and other hydrazides. The process was probably a direct oxidation since the presence of oxygen was required and sodium cyanide and sodium azide were inhibitors of the process. Both sensitive and resistant cells oxidised isoniazid. The cells also metabolised other hydrazides which are known to be less effective as tuberculostatic agents. From this it is suggested that the metabolism of hydrazides is not directly related to the mode of action of isoniazid but is more analagous to a detoxication mechanism.

In this connection it may be noted that *iso*nicotinic acid is excreted from the body after treatment with isoniazid (Cuthbertson and Ireland, 1953) and that the breakdown of isoniazid has been observed in a perfused organ (Takio Iwamoto, 1954) and in tissue slices (Garattini, Grassi, Mantegazza, Morvillo, Tommasini and Trabucchi, 1952).

The slower breakdown of isoniazid by killed cells was probably due to a chemical process. This did not appear to be due to metal catalysis but might perhaps be similar to the degradation of isoniazid in the presence of hemin (Albert and Rees, 1955).

The contrast between the rates of uptake of isoniazid by sensitive and resistant cells has been mentioned. Experiments with radioactive isoniazid, which will be described elsewhere, have detected only *iso*nicotinic acid in resistant cells and it seems likely that resistant cells metabolise isoniazid as fast as they take it up, while sensitive cells bind radioactivity more rapidly than they oxidise isoniazid. This may prove to be an explanation of the nature of the resistance to isoniazid.

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THE EFFECT OF PRECURSORS OF PYRIDINE NUCLEOTIDES ON POISONING BY CARBON TETRACHLORIDE

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SUMMARY.

The intraperitoneal injection of either nicotinic acid or DL-tryptophane, which are precursors of pyridine nucleotides, reduced considerably the death rate in rats subsequent to the administration by stomach tube of doses of carbon tetrachloride which were usually lethal. The greatest reduction in death rate was obtained when nicotinic acid or DL-tryptophane was given two days before CCl_4. Large doses of nicotinic acid injected five hours after CCl_4 was administered did not prevent deaths.

Nicotinic acid, which is a direct precursor of pyridine nucleotides, was more effective under the experimental conditions of this study in preventing deaths from CCl_4 poisoning than DL-tryptophane, which is a precursor of nicotinic acid.

Choline chloride, L-methionine, reduced glutathione, glucose and ethylenediaminetetraacetic acid did not appreciably reduce the death rate due to subsequent CCl_4 administration within the limits of this study.

In rats killed 20 hours after the administration of a lethal dose of CCl_4, there was a fall of more than 50 p.c. in the extinction coefficient at 260 mμ (E_{260}) of acid extracts of fresh liver homogenates or mitochondria prepared in 0·25 M sucrose compared with the figures for normal rats. The loss of material absorbing at 260 mμ in the homogenates was from the mitochondrial component.

Prophylactic injections of nicotinic acid maintained almost normal E_{260} values in acid extracts of liver homogenates of rats given CCl_4. Injection of nicotinic acid without a subsequent dose of CCl_4 did not result in an increase of E_{260} values above normal. It is probable, therefore, that nicotinic acid stimulates a rapid replacement of pyridine nucleotide lost from mitochondria as a result of CCl_4 administration. Greatest protection against the loss of E_{260} values was obtained when nicotinic acid was given two to three days before CCl_4.

The relationship between mitochondrial E_{260} values and the lethal effects of CCl_4 is discussed.

INTRODUCTION.

Christie and Judah (1954) have shown that the administration of carbon tetrachloride to rats disorganises the enzymic activity of liver mitochondria. The enzyme systems which are affected first are those which require pyridine nucleotides for electron transfer. After dosing rats with CCl_4 the inactivation of these

enzyme systems, evident in assays for enzyme activity *in vitro*, can be largely reversed by supplementing the reaction mixtures with the appropriate pyridine nucleotide. Further, when liver mitochondria from normal rats are pre-incubated with CCl_4 *in vitro* there is an inhibition of the tricarboxylic acid cycle activity similar in pattern to that resulting from the administration of CCl_4 to rats *in vivo* (Christie and Judah, 1954). From these studies, it appears likely that CCl_4 attacks mitochondria directly and thus causes an increased permeability of the mitochondrial membranes and a consequent loss of pyridine nucleotides and, therefore, an inactivation of the pyridine nucleotide-dependent enzyme systems.

Nicotinic acid has been shown to be a precursor of pyridine nucleotides by Preiss and Handler (1958a, 1958b). Similarly, tryptophane, which is a precursor of nicotinic acid, has been shown by Williams *et al.* (1950) and Chaloupka *et al.* (1957) to stimulate the synthesis of, and become incorporated into, pyridine nucleotides. The present study was undertaken to determine whether the administration of these precursors of pyridine nucleotides altered the susceptibility of rats to CCl_4 poisoning.

Materials and Methods.

Animals. Male Wistar rats weighing 150-210 gm. and fed a normal stock diet were used throughout the experiment.

Reagents. Carbon tetrachloride of reagent grade was further purified by distillation. Nicotinic acid, DL-tryptophane, L-methionine, reduced glutathione, choline chloride, disodium ethylenediaminetetraacetic acid, sucrose, glucose and inorganic reagents were commercial products of high purity.

Methods. The extinction coefficient at 260 mμ (E_{260}) of acid extracts of tissue may be taken as a measure of the concentration of the oxidized form of pyridine nucleotides, as Siekevitz and Potter (1953, 1955) have shown that the greatest proportion of the light absorption at this wavelength is due to oxidized pyridine nucleotides.

In order to measure the E_{260} values of liver, rats were stunned by a blow on the neck and killed by decapitation. Livers were then removed rapidly and immersed in ice-cold 0·25 M sucrose. Homogenates in 0·25 M sucrose of weighed portions of liver were prepared in glass homogenisers and mitochondria were isolated by differential centrifuging at 0° C. (Schneider, 1948). Mitochondria were re-suspended in 0·25 M sucrose. Immediately after the preparation of 10 p.c. W/V homogenates and of mitochondrial suspensions equivalent to 20 p.c. W/V of original fresh tissue, duplicate 0·25 ml. and 0·5 ml. aliquots of each were transferred to glass centrifuge tubes containing 5 ml. of 5 p.c. trichloroacetic acid at 0° C. After mixing by stirring with a glass rod the tube contents were allowed to stand at room temperature for 10 minutes and were then centrifuged at 2000 r.p.m. in an M.S.E. Multiplex centrifuge. Each of the supernatants was collected and the E_{260} was measured against a blank of 5 p.c. trichloroacetic acid, with a Beckman model D.U. spectrophotometer. Dry weights of homogenates were determined by extracting 20 ml. samples three times with a mixture of boiling ethanol and diethyl ether (3 to 1 V/V), to remove lipids, drying in an oven at 100° C. for 24 hours and then cooling to room temperature in a desiccator before weighing. Protein nitrogen of mitochondrial suspensions was determined by the micro-Kjeldahl method of McKenzie and Wallace (1954).

CCl_4 was administered undiluted into the stomach of unanaesthetised rats by a syringe attached to a tube. The dose used was 4 ml./kg. or 2·7 ml./kg. as indicated. These dose

rates were chosen after preliminary experiments. The larger dose was always fatal to control rats and was chosen as a severe test of the value of a prophylactic agent. The smaller dose was chosen as being just below the LD_{100} for the experimental conditions and was thus a considerable but lesser challenge to a prophylactic agent.

RESULTS.

Rats died from 22 to 90 hours after receiving either dose of CCl_4. Most deaths occurred from 48 to 72 hours after CCl_4 administration. Surviving rats were kept under observation for 21 days and were then destroyed.

Effect of precursors of pyridine nucleotides on CCl_4 toxicity.

Nicotinic acid. A dose of 4 ml. CCl_4/kg. proved fatal to 100 p.c. of control rats, but when this dose was given to rats on the last day of a course of three daily intraperitoneal injections of 50 mg. of nicotinic acid, the death rate was only 11·8 p.c. (Table 1). The 50 mg. doses of nicotinic acid were equally effective whether given as a neutral solution once daily or as an aqueous-acid solution split into two 25 mg. doses per day.

Table 1 shows that when the course of nicotinic acid administration was reduced to two days, 62·5 p.c. of eight rats succumbed to 4 ml. CCl_4/kg., compared with 100 p.c. of controls.

Complete protection was conferred on a group of nine rats given 2·7 ml. CCl_4/kg. by the intraperitoneal injection of a single dose of 50 mg. of nicotinic acid 48 hours previously (Table 1). However, with one 50 mg. dose of nicotinic acid, 4 ml. CCl_4/kg. given on the same day killed 88·9 p.c. and 2·7 ml. CCl_4/kg.

TABLE 1.

Effect of nicotinic acid and of DL-tryptophane on CCl_4 toxicity.

Procedure: Nicotinic acid (nic.ac.), DL-tryptophane (trypt.) and reduced glutathione (GSH) were injected intraperitoneally. CCl_4 was given by stomach tube.

Group	No. of rats	Treatment (mg.)			CCl_4 (ml/kg.) Day 3	Deaths	
		Day 1	Day 2	Day 3		No.	p.c.
Control	62	—	—	—	4·0	62	100·0
Nic.ac.	17	50	50	50	4·0	2	11·8
Nic.ac.	8	—	50	50	4·0	5	62·5
Nic.ac.	18	—	—	50	4·0	16	88·9
Nic.ac.+GSH	17	—	—	50+50	4·0	14	82·4
Control	47	—	—	—	2·7	40	85·1
Nic.ac.	20	—	—	50	2·7	18	90·0
Nic.ac.	9	50	—	—	2·7	0	0·0
Trypt.	16	50	50	50	2·7	5	31·3
Trypt.	9	—	50	50	2·7	6	66·7
Trypt.	8	—	—	50	2·7	6	75·0
Trypt.	9	50	—	—	2·7	4	44·5
Nic.ac.+Trypt.	9	—	—	50	2·7	9	100·0

killed 90·0 p.c. This latter dose of CCl_4 killed 85-94 p.c. of controls (Tables 1 and 2). There is no significant difference between the death rates of control rats given 2·7 ml. CCl_4/kg. and rats given this dose of CCl_4 and one dose of 50 mg. of nicotinic acid on the same day. The value of this single administration of nicotinic acid, coincident with CCl_4, was not enhanced by the simultaneous injection intraperitoneally of 50 mg. of reduced glutathione or 50 mg. of DL-tryptophane (Table 1).

The administration to each of nine rats of 250 mg. of nicotinic acid intraperitoneally five hours after giving 2·7 ml. CCl_4/kg. did not save the life of any.

Tryptophane. Table 1 shows that in comparison with nicotinic acid the intraperitoneal injection of DL-tryptophane was somewhat less effective in protecting against CCl_4 poisoning, but that there was a similar pattern for the two substances in relation to the time of administration and the protective effect. However, any comparison between the prophylactic value of nicotinic acid and DL-tryptophane can only be made within the limits of the experimental conditions, especially as an arbitrary dose rate of 50 mg. of either substance was used.

A course of DL-tryptophane for three days, at 50 mg./day, reduced the death rate to 31·3 p.c. subsequent to 2·7 ml./kg. CCl_4 administered by stomach tube on the same day as the third injection of DL-tryptophane, as compared with a death rate of 85-94 p.c. for control rats at this dose of CCl_4 (Tables 1 and 2). The death rate was 66·7 p.c. when 50 mg. of DL-tryptophane were given both on the day of CCl_4 administration and on the preceding day. The death rate of rats which received 50 mg. of DL-tryptophane on the same day as 2·7 ml. CCl_4/kg. was not significantly different from controls at this dose of CCl_4. As with nicotinic acid, a single dose of 50 mg. of DL-tryptophane had an appreciable protective effect only if given two days before CCl_4 (Table 1).

TABLE 2.

Effect of L-methionine, choline, glutathione, glucose and EDTA on CCl_4 toxicity.

Procedure: EDTA, reduced glutathione (GSH) and glucose were injected intraperitoneally in water. L-methionine in 0·5 N HCl was given by stomach tube. Choline chloride (I/P) was injected intraperitoneally and choline chloride (S/T) was given by stomach tube, both in water. CCl_4 was given by stomach tube.

Group	No. of rats	Treatment (mg.)			CCl_4 (ml./kg.) Day 3	Deaths	
		Day 1	Day 2	Day 3		No.	p.c.
Control	16	—	—	—	2·7	15	93·8
L-Methionine	10	200	200	200	2·7	7	70·0
Choline (I/P)	8	50	50	50	2·7	8	100·0
Choline (S/T)	8	250	250	250	2·7	8	100·0
GSH	8	50	50	50	2·7	5	62·5
GSH	8	—	50	50	2·7	7	87·5
GSH	7	—	—	50	2·7	5	71·4
Glucose	8	50	50	50	2·7	5	62·5
EDTA	10	50	25	25	2·7	10	100·0

Effect of other compounds on CCl_4 toxicity.

A number of other compounds, some of which have been suggested to be of therapeutic value in CCl_4 poisoning, were tested under the conditions described to determine their possible prophylactic value.

Table 2 shows that three 50 mg. doses intraperitoneally or three 250 mg. doses by stomach tube of choline chloride, or one 50 mg. and two 25 mg. doses intraperitoneally of disodium EDTA over three days respectively were of no protective value against the lethal affects of 2·7 ml. CCl_4/kg. Three 200 mg. doses by stomach tube of L-methionine, three or less 50 mg. doses intraperitoneally of reduced glutathione or three 50 mg. doses intraperitoneally of glucose were probably of no value in protecting against 2·7 ml. CCl_4/kg. under the conditions of testing.

Extinction coefficients at 260 mμ (E_{260}) of extracts of liver homogenates and mitochondria.

Homogenates and mitochondria of liver suspended in 0·25 M sucrose were prepared and extracted with 5 p.c. trichloroacetic acid, as described in the Methods section, to determine the effect of nicotinic acid and CCl_4 administration on the absorption of these preparations at 260 mμ, the absorption peak of oxidised pyridine nucleotides.

E_{260} of liver homogenates. The administration of CCl_4 resulted in the loss from liver homogenates of more than half the acid-extractable material which absorbed light at 260 mμ (Table 3). This loss was almost completely prevented by the prior intraperitoneal injection of 50 mg. of nicotinic acid on three successive days including the day of dosing with CCl_4 (Table 3).

Table 3 also shows that in rats given nicotinic acid only, the amount of acid-extractable material of liver which absorbed light at 260 mμ did not exceed that found in untreated control rats of the same sex and age.

TABLE 3.

E_{260} of acid extracts of liver homogenates.

Procedure: Trichloroacetic acid extracts of 0·25 ml. and 0·5 ml. duplicate samples of 10 p.c. liver homogenates were prepared as described in the Methods section. Group 1 received 50 mg. of nicotinic acid/day intraperitoneally for 3 days plus, where indicated, CCl_4 by stomach tube on the 3rd day. Rats were killed 20 hours after CCl_4 (2·7 ml./kg.) was given. Results have been corrected to 8 mg. of fat-free dry homogenate/sample.

Group	Treatment	No. of rats	E_{260}	
			Range	Mean
1a	Nicotinic acid	3	0·894—0·909	0·904
1b	Nicotinic acid + CCl_4	9	0·809—1·021	0·915
2	CCl_4	14	0·265—0·531	0·438
3	None	10	0·909—1·060	0·980

Effect of time of administration of nicotinic acid on E_{260}. Single intraperitoneal injections of 50 mg. of nicotinic acid were given to groups of rats to determine the most appropriate time of administration to protect against the loss of liver material absorbing light at 260 mμ which follows dosage with CCl_4.

In this experiment a single dose of nicotinic acid was found to be most effective in preventing the fall in liver E_{260} when given three days before CCl_4 (Table 4). An injection of nicotinic acid six days before CCl_4 reduced the loss somewhat, but an injection eight days before CCl_4 had no effect. Comparison of the data reported in Tables 3, 4 and 5 suggests that the optimal time to administer a single dose of nicotinic acid prophylactically is 2-3 days before giving CCl_4.

TABLE 4.

Effect of time of administration of nicotinic acid on E_{260}.

Procedure: Trichloroacetic acid extracts of 0·25 ml. and 0·5 ml. duplicate samples of 10 p.c. liver homogenates were prepared as described in the Methods section. Single, 50 mg. doses of nicotinic acid were injected intraperitoneally. All rats were given 2·7 ml. CCl_4/kg. by stomach tube on the 9th day of the experiment. Rats were killed 20 hours after CCl_4 was administered. Results have been corrected to 8 mg. of fat-free dry homogenate/sample.

Group	Time of treatment with nicotinic acid	No. of rats	E_{260}	
			Range	Mean
1	Day 1	5	0·241—0·322	0·282
2	Day 3	5	0·426—0·600	0·498
3	Day 6	5	0·742—0·902	0·818
4	Day 8	5	0·526—0·672	0·615
5	None	5	0·265—0·345	0·305

Effect of nicotinic acid on E_{260} of mitochondrial extracts. Groups of rats were given CCl_4 with and without prior treatment with nicotinic acid and the E_{260} of acid extracts of both liver homogenates and mitochondria were measured in order to determine—

i. whether, subsequent to CCl_4 administration, the loss from homogenates of material absorbing light at 260 mμ was from mitochondria; and

ii. whether nicotinic acid acted by maintaining in mitochondria the level of material absorbing at 260 mμ or by protecting this material from destruction after its release from mitochondria.

Table 5 shows that the decrease in the E_{260} of extracts of livers of rats given CCl_4 was in fact due to a loss of mitochondrial material.

Similarly, nicotinic acid was found to exert its effect at the mitochondrial level; when administered suitably, an almost normal E_{260} for mitochondrial extracts was maintained despite the administration of CCl_4 (Table 5).

TABLE 5.

Effect of nicotinic acid on E_{260} of mitochondrial extracts.

Procedure: Trichloroacetic acid extracts of 0·25 ml. and 0·5 ml. duplicate samples of 10 p.c. liver homogenates and of mitochondrial suspensions equivalent to 20 p.c. original homogenate were prepared as described in the Methods section. A single dose of 50 mg. of nicotinic acid was administered intraperitoneally 2 days before 2·7 ml. CCl_4/kg. were given by stomach tube to Group 1. Group 2 received 2·7 ml. CCl_4/kg. by stomach tube at the same time as Group 1. Rats were killed 20 hours after CCl_4 was administered. Results have been corrected to 8 mg. of fat-free dry homogenate/sample, and to 6 mg. of mitochondrial N/sample.

Group	Treatment	No. of rats	E_{260}			
			Homogenate		Mitochondria	
			Range	Mean	Range	Mean
1	Nicotinic acid + CCl_4	4	0·738—0·840	0·801	0·284—0·316	0·304
2	CCl_4	4	0·312—0·501	0·427	0·127—0·172	0·150
3	None	4	0·930—1·060	0·992	0·367—0·381	0·372

DISCUSSION.

The death rate in rats given usually lethal doses of carbon tetrachloride has been greatly reduced by the prior administration of precursors of pyridine nucleotides. Both nicotinic acid, which is a direct precursor of pyridine nucleotides and DL-tryptophane, which is a precursor of nicotinic acid, exert a prophylactic effect against poisoning by CCl_4. Each of these compounds stimulates the synthesis of pyridine nucleotides when administered to animals (Williams *et al.*, 1950; Chaloupka *et al.*, 1957; Preiss and Handler, 1958a, 1958b).

Nicotinic acid is more effective in preventing CCl_4 poisoning than DL-tryptophane in the dosage used. However, the intraperitoneal injection of either to rats on each of the two days preceding and on the day of CCl_4 administration greatly reduced the death rate. A single dose of either nicotinic acid or DL-tryptophane is most effective when given two days before CCl_4. However, a single dose of either substance on the day of CCl_4 administration does not protect significantly. Large doses of nicotinic acid given five hours after CCl_4 did not prevent deaths.

Other compounds, which are not known to affect the synthesis of pyridine nucleotides but some of which have been claimed to influence the course of CCl_4 poisoning, were tested as prophylactic agents. Under the experimental conditions of the present study, none of these compounds, L-methionine, choline, reduced glutathione, glucose or EDTA, was found to be of any appreciable value.

Eddy (1945) and Shiels (1958) claimed that L-methionine was a useful therapeutic agent in CCl_4 poisoning, and Patwardhan *et al.* (1953, 1954) that L-methionine or glutathione favourably influenced regenerative changes in CCl_4-poisoned livers. While L-methionine and glutathione may hasten the recovery

of liver parenchyma from non-lethal doses of CCl_4, they did not increase significantly the resistance of rats to lethal doses in the present study.

Choline, which was shown by Barrett *et al.* (1939) to reduce liver damage resulting from chronic poisoning with CCl_4 and by Christie and Judah (1954) to delay the onset of mitochondrial dysfunction, afforded no protection against a lethal dose of CCl_4. Calvert and Brody (1958) were unable to verify the protective effect of choline administration on mitochondrial function.

Again, although Calvert and Brody (1958) have observed that after the simultaneous administration of EDTA intraperitoneally and of CCl_4 by stomach tube there was some protection against the loss of activity of liver mitochondrial enzyme systems of rats killed 20 hours later, in the present study EDTA was of no prophylactic value against lethal doses of CCl_4.

Finally, glucose, injected into rats to determine whether increased carbohydrate intake might reduce susceptibility to CCl_4 poisoning, was of no significant value as a prophylatic agent under the conditions of this study.

It seemed likely that nicotinic acid or tryptophane exercised a protective effect either by stimulating the synthesis of pyridine nucleotides, resulting in higher concentrations within mitochondria, or that the precursors provided for a more rapid replacement of any pyridine nucleotides which might be lost due to the action of CCl_4. To investigate these possibilities, the extinction coefficient at 260 mμ (E_{260}) of acid extracts of liver was measured. As a large part of the optical absorption at 260 mμ by acid-soluble material of cells is due to the oxidized forms of pyridine nucleotides, the E_{260} of acid extracts may be taken as a measure of the concentration of oxidized pyridine nucleotides (Siekevitz and Potter, 1953, 1955).

In acid extracts of liver homogenates prepared from rats killed 20 hours after the administration of CCl_4, there was a decrease of more than 50 p.c. in the E_{260}. This decrease was almost completely prevented by a course of three daily injections ending on the day of administration of CCl_4, and very largely so by a single dose of nicotinic acid given two to three days prior to administration of CCl_4.

The results indicate that the maintenance of E_{260} values after CCl_4 administration is due to rapid replenishment of mitochondrial pyridine nucleotides rather than to an actual increase in concentrations within the mitochondria prior to giving CCl_4, because a course of nicotinic acid alone does not increase the levels of pyridine nucleotides above normal.

The decrease in acid-soluble material absorbing at 260 mμ from liver homogenates was found to be due to loss of this material from the mitochondria of the homogenates. As no attempt was made to inhibit pyridine nucleotidase activity, pyridine nucleotides would be destroyed as they leaked from mitochondria (*cf.* Kaufman and Kaplan, 1959); this would account for the total fall in E_{260} of homogenates of whole liver.

Recknagel and Anthony (1959) and Share and Recknagel (1959), have sug-

gested that in the livers of rats which have received CCl_4, the loss of pyridine nucleotides from mitochondria and the inactivation of pyridine nucleotide-dependent dehydrogenases may have occurred not *in vivo* but during the preparation of enzymes and assay of enzymic activity *in vitro*; particularly during the equilibration period before taking readings of oxygen uptake at 38° C. The present study shows that at least the major loss of pyridine nucleotides does not occur when mitochondria are suspended in the respiratory medium used by Christie and Judah (1954) and brought to 38° C., because all manipulations from the killing of the rats to the addition of homogenates or mitochondria in 0·25 M sucrose to 5 p.c. trichloroacetic acid were carried out at 0-1° C.

The prophylactic value of precursors of pyridine nucleotides against the lethal effects of CCl_4 and against the loss of material absorbing light at 260 mμ from mitochondria following CCl_4 administration has been demonstrated. This finding provides strong support for Christie and Judah's evidence (1954) that CCl_4 disorganises mitochondria *in vivo* by causing increased permeability of mitochondrial membranes, the loss of mitochondrial pyridine nucleotides and the inactivation of enzyme systems which are dependent upon pyridine nucleotides for electron transport. When the concentration of mitochondrial pyridine nucleotides as indicated by E_{260} values was maintained at an approximately normal level by injections of precursors an associated protection of rats from the lethal effects of CCl_4 was demonstrated.

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NITRATE ADAPTATION BY AGEING SUSPENSIONS OF *PS. AERUGINOSA*

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SUMMARY.

The rate of adaptation of the enzyme nitrate reductase of *Ps. aeruginosa* increased sharply to a peak 12-14 hours after the stationary growth phase commenced. The rate later returned to the initial value. No similar increase in the adaptation rate for the nitrate reducing enzyme system as a whole was observed.

INTRODUCTION.

Hegarty (1939) reported extensive variations in the rate at which ageing suspensions of *Str. lactis* adapted to a number of carbohydrates. The most rapid rate of adaptation was observed when the cells were in the logarithmic growth phase. Pinsky and Stokes (1952) also investigated the effect of ageing on the rate of enzymatic adaptation, but were unable to confirm the finding that maximum adaptation occurred during the logarithmic growth phase. Both the formic hydrogenase and the nitrate reductase of *E. coli* adapted at the maximum rate when the cells were in the stationary phase. Subsequently, Arima *et al.* (1955) and Maruyama and Mitui (1958) reported slow enzymatic adaptation during the logarithmic phase compared with cells in the stationary phase. On the other hand, Ravin (1953) showed that *A. aerogenes* slowly lost the ability to adapt to citrate during the stationary and decline phases.

The variation in the adaptation rate by ageing cells was thus firmly established, but the stage of growth at which maximum adaptation occurred seemed to vary widely. The present experiments were carried out to see whether the variation in the rate of adaptation of a single enzyme (nitrate reductase) was similar to that for the nitrate reducing enzyme sequence as a whole.

MATERIALS AND METHODS.

Ps. aeruginosa NCTC 6750 was grown on digest agar slopes for 18 hours at 37° C. A little of the growth was inoculated into 80 ml. of digest broth and incubated at 37° C. on a constant speed shaking machine for 12 hours. The cells were spun down aseptically and washed once with sterile saline. The suspension was standardised turbidimetrically. Sufficient of this suspension was pipetted aseptically into 200 ml. of digest broth in a two litre Erlenmeyer flask to give an initial population of 10^8 cells per ml. The culture was shaken for 26 hours and samples were removed for testing every two hours. Viable counts

were made by the spot plate method of Miles and Misra (1938), while total counts were carried out on each sample by the method of Williams (1952).

Each sample was centrifuged at 5,000 r.p.m. for 20 minutes and the cells washed with sterile M/15 phosphate buffer (pH 7·4) and standardised turbidimetrically to 5×10^9 cells per ml. The nitrate reductase adaptation of each suspension was determined by the method of Pollock (1946). Nitrite was determined by the method of Rider and Mellon (1946) after precipitation of the cells with copper sulphate and magnesium carbonate (Lees and Quastel, 1946). The adaptation rate expressed as µg. nitrite-N produced per hour per 10^9 viable cells was plotted against the age of the cells in hours.

The adaptation of the nitrate reducing enzyme system as a whole was measured in Warburg manometers under an atmosphere of oxygen-free nitrogen. Each cup contained two ml. of suspension, 0·1 ml. of 0·2 M sodium lactate and 0·2 ml. of 10 p.c. casein hydrolysate. The side arm contained 0·1 ml. of 0·2 M sodium nitrate and the CO_2 was absorbed in 0·2 ml. of 10 p.c. KOH. Nitrogen gas evolution was measured over a period of four hours and the adaptation rate, expressed as µl. nitrogen gas evolved per hour per 10^9 viable cells, was plotted against the age of the cells to give an adaptation curve.

The average length of the cells in each suspension was determined by measuring 50 random cells. The osmic acid fixed cells were stained by Gram and a suitable field was photographed on Ilford Pan F film. The film was projected onto a screen marked with a ruled grid; the cells were measured in mm. and the mean calculated.

RESULTS.

Unadapted suspensions of *Ps. aeruginosa* incubated in the presence of nitrate, a suitable energy source (0·2 M sodium lactate) and casein hydrolysate, showed a lag of 15-30 minutes before nitrite could be detected in the medium (Fig. 1). The length of the lag was minimal (15 minutes) during the stationary phase and longest in the early logarithmic phase. Thereafter, nitrite formation was linear until 90 to 180 minutes (Fig. 1) when adaptation of the nitrite reductase resulted in the rapid removal of the nitrite from the medium. The adaptation curve (Fig. 2) clearly demonstrated the considerable and rapid increase in the rate of adaptation of the nitrate reductase during the stationary phase (18-20 hours). However, this rapid increase was short-lived and the curve had returned to its initial value by the 26th hour. The logarithmic phase suspensions had only 5-10 p.c. of the activity of the 20-hour suspension (late stationary phase). The two-fold increase in activity during the lag phase (0 to 2 hours) was found to coincide with an equivalent increase in the mean length of the cells (Fig. 2). Once active cell division commenced the length of the rods decreased rapidly and the adaptation rate returned to its initial value. Thus the increase was probably simply a reflection of the change in cell volume rather than a real alteration in enzyme activity.

The length of the lag before nitrogen gas evolution was detected manometrically was approximately 60 minutes for the 0, 2, 4 and 6 hour old suspensions (Fig. 3). The 8 and 12 hour suspensions also showed a lag of only 60 minutes which decreased to 30 minutes for the 14 to 24 hour cells. The 18 hour

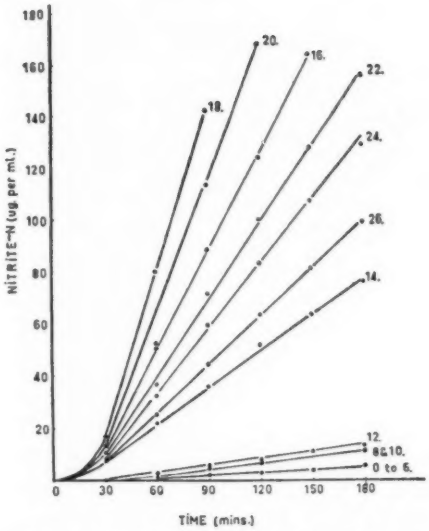

Fig. 1. Nitrite-N production by ageing suspensions of *Ps. aeruginosa*. The figures on the graph refer to the age (in hours) of the suspension.

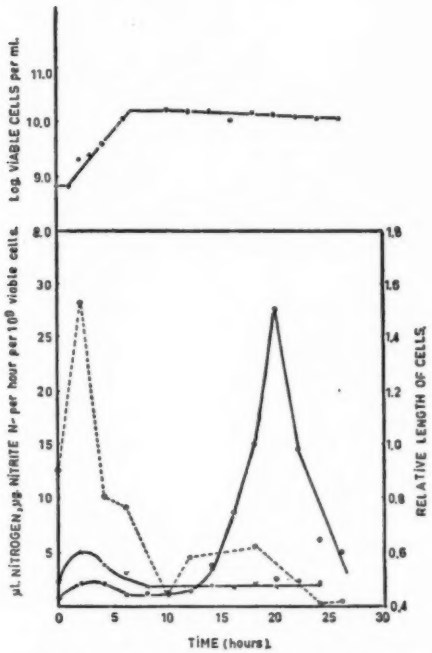

Fig. 2. The rate of nitrate adaptation by ageing suspensions of *Ps. aeruginosa*. ●——● Viable growth curve. ○——○ Adaptation curve for nitrate reductase. ×——× Adaptation curve calculated from the rate of nitrogen gas evolution. ○---○ Relative average length of the ageing organisms.

suspension showed the shortest lag which in one replicate experiment was only 15 minutes. Once the lag was completed, the rate of gas evolution was linear until the nitrate supply was exhausted. The rate of gas production per hour per 10^9 viable cells was plotted against the age of the suspensions to give the adaptation curve shown in Fig. 2. After the slight increase in activity during the lag and early logarithmic growth phases, the adaptation rate remained stationary throughout the remainder of the experiment. This was confirmed by two duplicate experiments.

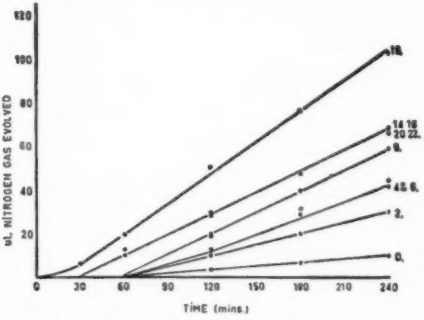

Fig. 3. Nitrogen gas evolution by ageing suspensions of *Ps. aeruginosa* measured under anaerobic conditions in Warburg manometers. The figures on the graph refer to the age (in hours) of the suspension.

DISCUSSION.

In agreement with the findings of most earlier workers, adaptation of both the nitrate reductase and the nitrate reducing enzyme system as a whole was found to be minimal during the early logarithmic phase. However, the considerable increase in nitrate reductase activity in the late stationary phase, without any corresponding increase in the overall activity of the nitrate reducing system was unexpected. The difference in adaptive ability of the various enzymes in the one system would seem to exclude the possibility that an increase in lactic dehydrogenase activity was responsible for the sudden rise in the nitrate reductase activity. Such an increase in dehydrogenase activity would be expected to affect the nitrate reducing system as a whole rather than only the first component.

The increased rate of adaptation of the nitrate reductase in the 16-24 hour period led to a temporary accumulation of nitrite in the medium. Quantitative nitrite estimations carried out on anaerobically incubated nitrate broth cultures of *Ps. aeruginosa* disclosed the presence of traces of nitrite at a time corresponding to the peak in nitrate reductase activity (Collins, unpublished experiments). The accumulation of the nitrite in growing cultures indicated that the nitrate reductase activity of the organism was greatly increased relative to the later enzymes of the reduction pathway. The estimation of the rate of nitrate adaptation of the organism in terms of the overall reduction to nitrogen gas gave no indication of the extensive variations in the activity of at least one component of the system. The reason for the variation in the adaptation rate of these closely associated enzymes was not obvious.

The adaptation of ageing cells of *Ps. aeruginosa* was made in terms of viable cells rather than dry weight or total nitrogen because the viability of the organism often decreased rapidly during the experiment. Since the dead cells were unable to adapt to nitrate the use of total protoplasma as a basis for enzyme activity introduced considerable errors, especially in the older suspensions.

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